Pekka Peroja

OXIDATIVE STRESS IN DIFFUSE LARGE B-CELL LYMPHOMA AND FOLLICULAR LYMPHOMA, AND TP53 MUTATIONS AND TRANSLOCATIONS OF MYC, BCL-2 AND BCL-6 IN DIFFUSE LARGE B-CELL LYMPHOMA
PEKKA PEROJA

OXIDATIVE STRESS IN DIFFUSE LARGE B-CELL LYMPHOMA AND FOLLICULAR LYMPHOMA, AND TP53 MUTATIONS AND TRANSLOCATIONS OF MYC, BCL-2 AND BCL-6 IN DIFFUSE LARGE B-CELL LYMPHOMA

Academic dissertation to be presented with the assent of the Doctoral Training Committee of Health and Biosciences of the University of Oulu for public defence in Auditorium 7 of Oulu University Hospital, on 20 April 2018, at 12:00 noon.
Abstract

Diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL) are two of the most common lymphomas in the Western world. DLBCL is an aggressive disease with a good response to treatment; about 75% of patients achieve permanent remission after first-line treatment. In patients with relapses or primary refractory disease, prognosis is dismal; only 10–20% of them can be cured, even with aggressive treatments. FL is an indolent lymphoma with a very good response to treatment and slow progression. Median survival with modern treatments is over 15 years. Nevertheless, some patients have short remissions and succumb to disease. Oxidative stress, TP53 mutations, and translocations of MYC, Bcl-2 and Bcl-6 have been linked in many neoplasms to aetiology and poor prognosis.

This thesis concerns oxidative stress and redox-state-regulating enzymes in DLBCL and FL, and TP53 mutations and translocations of MYC, Bcl-2 and Bcl-6 in DLBCL. High expression levels of the antioxidant enzyme thioredoxin and a marker of oxidative stress, nitrotyrosine, were related to poor prognosis in DLBCL. In FL, high-level expression of peroxiredoxin was associated with good prognosis. TP53 mutations in specific regions LSH and L3 and concurrent translocation of Bcl-2 were associated with poor prognosis in DLBCL. Not all TP53 mutations predicted survival. High expression levels of Bcl-2 and MYC were associated with poor prognosis in DLBCL.

Based on the results presented here, antioxidant function may have protective roles, but also may cause resistance to treatment. TP53 mutations have prognostic roles in DLBCL, but should be further defined. Novel therapies could be developed in connection with these mechanisms.

Keywords: diffuse suurisoluinen B-solulymfooma, ennuste, folliculaarinyn lymfooma, immunoelektronimikroskopia, immunohistokemia, lymfooma, oksidatiivinen stressi, thioredoksiini, TP53 mutaatiot, translokaatioanalyysit
Peroja, Pekka, Oksidatiivinen stressi diffuusissa suurisoluisessa B-solulymphoomassa ja follikulaarisesse lymphoomassa ja TP53 mutaatiot ja MYC, Bel-2 ja Bcl-6 translokaatiot diffuusissa suurisoluisessa B-solulymphoomassa.

Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta; Medical Research Center; Oulun yliopistollinen sairaala

Acta Univ. Oul. D 1458, 2018
Oulun yliopisto, PL 8000, 90014 Oulun yliopisto

Tiivistelmä

Diffuusi suurisoluinen B-solulymphooma (DLBCL) ja follikulaarinen lymphooma (FL) ovat kaksi yleisintä lymphoomaa länsimaissa. DLBCL on aggressiivinen syöpä, joka reagoi hyvin hoitoihin, jopa 75 % paranee. Kuitenkin potilailta, joilla syöpä uusiutuu hoitojen jälkeen tai etenee hoidon aikana, on erittäin huono ennuste, noin 10-20 % näistä potilaista voidaan parantaa. FL on hyväennusteinen lymphooma, joka yleensä reagoi hyvin hoitoihin. Mediaani elossaoloihin kaikilla FL potilailla on yli 15 vuotta taudin toteamisesta. Osalla potilaista FL kuitenkin on aggressiivisempi. Oksidatiivinen stressin, TP53- mutaatioiden, MYC, Bcl-2 ja Bcl-6 -translokaatioiden on todettu olevan huonoon ennusteen yhteydessä olevia tekijöitä monissa syövissä, kuten lymphoomissa.


Tulosten perusteella solujen hapetus-pelkistystilaa säätelivillä entsyymeillä voi olla dualistinen rooli, osittain suojeleva ja osittain vahingoittava lymphoomissa. TP53 -mutaatioilla voi olla ennusteellista merkitystä, mutta tämä vaatii lisää tutkimuksia.

Asiasanat: diffuse large B-cell lymphoma, follicular lymphoma, immunoelectronmicroscopy, immunohistochemistry, lymphoma, oxidative stress, prognosis, thioredoxin, TP53 mutations., translocation analysis
Acknowledgements

This study was carried out at the University of Oulu; Oulu University Hospital, Department of Oncology and Radiotherapy and Medical Research Unit Oulu, Oulu Finland.

First, I would like to acknowledge, my supervisors, Docent Outi Kuittinen, Professor Taina Turpeenniemi-Hujanen and Docent Peeter Karihtala. Outi was first to introduce me to the world of lymphomas and her constant support has been invaluable though the journey. Taina has made all the projects possible and her vast knowledge of research has opened new doorways. Peeter has accumulated vast knowledge of oxidative stress and his contributions are immesurable.

I thank my co-authors in their valued roles in these projects. I would like to thank especially docent Kirsi-Maria Haapasaari, for her vital role in the evaluation of hispopathology. I thank Mrs Anne Bisi and Mrs Erja Tomperi for their invaluable work in the laboratory.

I am most grateful to my reviewers, Docent Veli-Matti Kosma and Docent Timo Paavonen, for their careful concideration of my thesis and valuable advice. I thank also my supervisors Docent Jussi Koivunen, Docent Arja Jukkola and Docent Marjaana Säily for support and encouragement.

And last but not least I thank my family and friends for their constant support. Especially I thank Nina for helping me through rough patches.

This work was funded by The Finnish Medical Foundation Duodecim.

Oulu, December 2017

Pekka Peroja
Abbreviations

8-OHdG  8-hydroxy-2’-deoxyguanosine
ABC  activated B-cell
BCR  B-cell receptor
BL  Burkitt’s lymphoma
CHOP  cyclophosphamide, doxorubicin, vincristine and prednisone
COEP  cyclophosphamide, epirubicin, vincristine and prednisone
CHOEP  cyclophosphamide, doxorubicin, vincristine, etoposide and prednisone
CLL  chronic lymphocytic leukaemia
CNS  central nervous system
DFS  disease free survival
DSS  disease specific survival
COO  cell of origin
DH  double hit
DLBCL  diffuse large B-cell lymphoma
EBV  Epstein–Barr virus
ECOG  Eastern Cooperative Oncology Group
*e.g.*  exempli gratia
FISH  fluorescence *in situ* hybridisation
FL  follicular lymphoma
FLIPI  follicular lymphoma international prognostic index
GCB  germinal centre B-cell
GCL  glutamate-cysteine ligase
GEP  gene expression profiling
GPx  GSH peroxidase
GSH  thiol-reduced glutathione
GSSG  disulphide-oxidized glutathione
H$_2$O$_2$  hydrogen peroxide
HBV  hepatitis B virus
HCV  hepatitis C virus
HIV  human immunodeficiency virus
HL  Hodgkin’s lymphoma
HLA  human leukocyte antigen
HR  hazard ratio
LSH  loop-sheet helix
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.e.</td>
<td>id est</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IPI</td>
<td>international prognostic index</td>
</tr>
<tr>
<td>IVLBCL</td>
<td>primary intravascular large B-cell lymphoma</td>
</tr>
<tr>
<td>MALT</td>
<td>mucosa-associated lymphoid tissue/extranodal marginal zone B cell</td>
</tr>
<tr>
<td>MCL</td>
<td>mantle cell lymphoma</td>
</tr>
<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor kappa-light-chain-enhancer of B-cells</td>
</tr>
<tr>
<td>NHL</td>
<td>non-Hodgkin’s lymphoma</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer cell</td>
</tr>
<tr>
<td>NOS</td>
<td>not otherwise specified</td>
</tr>
<tr>
<td>OH⁻</td>
<td>hydroxide</td>
</tr>
<tr>
<td>OS</td>
<td>overall survival</td>
</tr>
<tr>
<td>PARP1</td>
<td>protein poly(ADP-ribose) polymerase 1</td>
</tr>
<tr>
<td>PCNSL</td>
<td>primary central nervous system lymphoma</td>
</tr>
<tr>
<td>PMBL</td>
<td>primary mediastinal B-cell lymphoma</td>
</tr>
<tr>
<td>Prx</td>
<td>peroxiredoxin</td>
</tr>
<tr>
<td>PTL</td>
<td>primary testicular lymphoma</td>
</tr>
<tr>
<td>R</td>
<td>rituximab</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>T/HRLBCL</td>
<td>T-cell/histiocyte-rich large B-cell lymphoma</td>
</tr>
<tr>
<td>TAD</td>
<td>transactivation domain</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>TFH cells</td>
<td>follicular helper T-cells</td>
</tr>
<tr>
<td>Trx</td>
<td>thioredoxin</td>
</tr>
<tr>
<td>UA</td>
<td>uranyl acetate</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
</tbody>
</table>
List of original publications

This thesis is based on the following publications, which are referred to throughout the text by their Roman numerals:


Article I was included in Thesis titled a translational study on the roles of redox molecules, cell cycle regulators and chemokine receptors as prognostic factors in diffuse large B-cell lymphoma by Anna Kaisa Pasanen.
Contents

Abstract
Tiivistelmä
Acknowledgements 7
Abbreviations 9
List of original publications 11
Contents 13
1 Introduction 15
2 Review of the literature 17
  2.1 Lymphomas .............................................................. 17
      2.1.1 Aetiology ......................................................... 18
      2.1.2 Epidemiology .................................................. 19
      2.1.3 Treatment ....................................................... 19
  2.2 Diffuse Large B-cell lymphoma ................................... 20
      2.2.1 Aetiology and epidemiology .............................. 20
      2.2.2 Subtypes ....................................................... 21
      2.2.3 Biological variance ......................................... 23
      2.2.4 Prognostic tools ............................................. 26
      2.2.5 Treatment ....................................................... 27
  2.3 Follicular lymphoma ................................................ 28
      2.3.1 Aetiology and epidemiology .............................. 29
      2.3.2 Biology ........................................................ 29
      2.3.3 Disease transformation .................................... 31
      2.3.4 Clinics .......................................................... 33
      2.3.5 Treatment ....................................................... 33
  2.4 Double-hit lymphomas .............................................. 35
  2.5 Oxidative stress ....................................................... 35
      2.5.1 Oxidative stress markers ................................ 37
      2.5.2 Manganese superoxide dismutase ................. 39
      2.5.3 Glutathione-related antioxidant defence .......... 41
      2.5.4 Thioredoxins ............................................... 42
      2.5.5 Peroxiredoxins ............................................ 44
  2.6 Tumour protein 53 .................................................. 46
      2.6.1 Structure ........................................................ 47
      2.6.2 Function ........................................................ 49
      2.6.3 Mutations ....................................................... 51
2.6.4 Lymphomas and haematological malignancies ....................................... 52
2.7 Transcription regulators ............................................................................... 53
  2.7.1 MYC ..................................................................................................... 53
  2.7.2 Bcl-2 .................................................................................................. 55
  2.7.3 Bcl-6 .................................................................................................. 56
3 Aims of the present study ............................................................................. 59
4 Materials and methods .................................................................................. 61
  4.1 Patient population and methods (I–III) .................................................... 61
    4.1.1 Details of collected information (I–III) .................................... 61
  4.2 Immunohistochemistry (I–III) .................................................................. 63
  4.3 Immunoelectron microscopy (II) ............................................................... 64
  4.4 Fluorescence in situ hybridisation (III) ..................................................... 65
  4.5 Microdissection (III) ................................................................................. 65
  4.6 TP53 mutation analysis (III) ...................................................................... 66
  4.7 Statistical analysis (I–III) ........................................................................ 66
  4.8 Ethical aspects .......................................................................................... 67
5 Results ........................................................................................................... 69
  5.1 Oxidative stress staining patterns in DLBCL (I) ........................................ 69
  5.2 Oxidative stress staining analysis in DLBCL (I) ....................................... 69
  5.3 Oxidative stress staining patterns in FL (II) .............................................. 71
  5.4 Oxidative stress staining analysis in FL (II) ............................................. 75
  5.5 TP53 mutations (III) ................................................................................ 76
  5.6 Translocation and immunohistochemistry of MYC, Bcl-2 and Bcl-6 (III) ........................................................................................................... 77
  5.7 Concurrent analysis of TP53 mutation, translocation and
    immunohistochemistry (III) ........................................................................ 77
6 Discussion ..................................................................................................... 79
  6.1 Role of oxidative stress in DLBCL ............................................................ 79
  6.2 Redox enzymes and oxidative stress in FL .............................................. 80
  6.3 TP53 mutations, MYC, Bcl-2 and Bcl-6 in DLBCL .................................... 81
  6.4 Limitations in material and methods .......................................................... 83
  6.5 Clinical implications .................................................................................. 84
7 Summary ...................................................................................................... 87
References ....................................................................................................... 89
Original Publications ....................................................................................... 131
1 Introduction

Lymphomas are malignant neoplasms originating from immunological cells. They can originate from several different cell types, but the most common are B-cell lymphomas. Lymphomas have varying disease profiles; some are “very low progressive”, i.e. indolent lymphomas, and some are very aggressive, with dismal prognosis. The two most common B-cell lymphomas are diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL).

DLBCL is the most common lymphoma in the Western world. It is an aggressive disease, but very often susceptible to treatment. Not all DLBCLs behave similarly and several different categories of DLBCL have been created. There is no way to distinguish those patients with aggressive disease modalities currently. Follicular lymphoma is the second most common lymphoma and is usually a very indolent disease. Median overall survival (OS) with modern treatments after diagnosis is thought to be over 15 years. Transformation of FL into more aggressive lymphoma is possible. To identify patients that would require more effective first-line treatment is crucial to improve outcome in cases of these two types of lymphoma.

Oxidative stress is a common denominator for all cells that require oxygen. It is an integral part of normal cell function and it is tightly controlled in the normal state by antioxidants and redox-state-regulating enzymes. Decreased function of redox-state enzymes or increased oxidative stress leads to cellular damage, and damage leads to apoptosis or even carcinogenesis. In lymphomas, oxidative stress and redox-state enzymes are thought to play a role as in all neoplasms, but that current role is unknown.

Tumour protein 53 (p53) is one of the most important proteins in the protection of DNA. Some consider it to be mutated or non-functional in 50% of all neoplasms. In DLBCL TP53 mutations are more uncommon than in solid neoplasms, but TP53 mutations in DLBCL seem to be associated with inferior survival in patients. MYC is a highly potent transcriptional activator and repressor that controls growth and proliferation. MYC is translocated in some DLBCLs. Bcl-2 is an anti-apoptotic protein that does not increase proliferation, but stops apoptosis. Bcl-6 is a transcriptional factor that is also anti-apoptotic and protects cells against premature apoptosis. Translocations of MYC, Bcl-2 and Bcl-6 cause constitutive activation of these factors and are implicated in poor survival in cases of DLBCL.
The present study was conducted to evaluate oxidative stress and redox enzymes in DLBCL and FL and to uncover more knowledge of TP53 mutations and translocations, and increased protein expressions of MYC, Bcl-2 and Bcl-6 in DLBCL.
2 Review of the literature

2.1 Lymphomas

Lymphomas are malignant neoplasms that arise from lymphoid cells. Lymphomas are a heterogeneous group of neoplasms, as some are very aggressive and others are of an indolent nature. Before better understanding of the biological nature of lymphomas, they were coarsely classified into two categories, i.e. Hodgkin’s lymphoma (HL) and non-Hodgkin’s lymphoma (NHL). HL was first reported in an article by Thomas Hodgkin in 1832. This article described autopsy findings in seven patients with enlarged spleens, and lymph nodes without inflammation or other pathological findings that would explain the enlargement (Hodgkin, 1832), but even before that, Marcello Malpighi, the so-called father of microscopic anatomy, had described a condition with similar characteristics in 1666 (Stone, 2005). The World Health Organisation (WHO) classification of Tumours of Haematopoietic and Lymphoid Tissues 2016 is the most current classification. In the current classification lymphomas are divided into over 80 different subtypes. The first version of this WHO classification was published in 2001 (Campo et al., 2011; Swerdlow et al., 2016).

Lymphomas can be divided into NHL and HL on the basis of historical classification. NHL can be further divided into three groups based on the original cell type, i.e. natural killer- (NK), T-cell- and B-cell-derived lymphomas. HLs originate from B-cells. The most common lymphomas are B-cell-derived lymphomas and their disease patterns vary; some are very indolent in nature and require minimal treatment. Others are very aggressive, without treatment leading to death a few months after diagnosis. T-cell and NK-cell lymphomas are less common but more often than not aggressive diseases. The most common NHL lymphomas are diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), chronic lymphocytic leukaemia (CLL), mantle cell lymphoma (MCL), mucosa-associated lymphoid tissue/extranodal marginal zone B cell (MALT) lymphoma and Burkitt’s lymphoma (BL). DLBCL and FL will be described in greater detail in following chapters. DLBCL is the most common of B-cell lymphomas and FL is the second most common. About one third of newly diagnosed NHLs in Western countries are DLBCL and about one fifth are FL. The third most common is the HL variant lymphoma. This lymphoma type is characterized by its large amount of non-neoplastic cells, and only approximately
2–3% of cells in the neoplastic tissue are cancerous Hodgkin–Reed–Sternberg cells (van Leeuwen et al., 2014).

### 2.1.1 Aetiology

The exact aetiology of lymphomas is largely unclear. Several viral infections have been linked to an increased incidence of lymphoma. There has been some evidence of causality as regards HL and Epstein–Barr virus (EBV) in young adults (Hjalgrim et al., 2003), but other oncogenic factors or mutations are also required, *i.e.* chronic infection (Vockerodt, Cader, Shannon-Lowe, & Murray, 2014) and/or oxidative stress (X. Chen, Kamranvar, & Masucci, 2016). Human immunodeficiency virus (HIV) and NHL have a clear association, with increased death from lymphoma among the afflicted, but this might be due to lower immunological competence (Rios, 2014). Hepatitis C virus (HCV) is also associated with increased NHL incidence and the pooled risk factor (from several studies) is about 2.5. In addition, HCV has been linked with increased oxidative stress and this might be one of the explanatory causes of the association between lymphoma and the virus (Ivanov, Bartosch, Smirnova, Isaguliants, & Kochetkov, 2013). NHL is associated with hepatitis B virus (HBV), with a pooled odds ratio of about 2.56. The exact nature of this connection is unknown, but it is thought that there is a causal relationship between hepatitis and NHL, with the hepatitis virus being the oncogenic component in the development of NHL (Marcucci & Mele, 2011). In some studies the link between hepatitis and NHL has been even higher; serum HBV positivity correlated with NHL, showing a hazard ratio (HR) of 3.56 (CI 95% 1.37–9.18) in a Japanese population-based cohort study (Abe et al., 2015). In a recent study, NHL and simian virus 40 were linked together in comparison of NHL and two other neoplasms (Tognon et al., 2015). There is clear evidence of a link between immunodeficiency or chronic disease, and HL (Bruni et al., 2014; Gu et al., 2014; Kristinsson et al., 2015). Environmental and dietary reasons have also been suggested, *e.g.* pesticides (Alavanja et al., 2014; Colt et al., 2005), occupation, tobacco, alcohol consumption (Morton et al., 2014) and red meat consumption (Fallahzadeh, Cheraghi, Amoori, & Alaf, 2014). Other unknown environmental factors may play a part in lymphomagenesis, as a few residential areas have been identified with a higher incidence in spatial-temporal analysis (Nordsborg et al., 2015; Wheeler et al., 2011). It is most likely that development of lymphoma results from a combination of genetic and risk factors that remain largely undiscovered.
2.1.2 Epidemiology

The incidence of lymphomas is rising by about 3% annually, being a rate which is higher than that associated with most other neoplasms. This rise is mostly due to an increase in NHL cases. The incidence of HL has remained steady and prevalence is constant. The NHL increase has especially been noted in Western countries. The incidence of NHLs is rising partly because of longer life-spans of populations. A longer life-span increases the risk of malignancies overall. However, there might be unidentified risk factors that increase susceptibility to NHLs overall.

There are large variations in the incidence of HL and NHL in different parts of the world. For example, BL is a common lymphoma in equatorial Africa (Stefan & Lutchman, 2014), as are T-cell lymphomas in Asia (Armitage, 2015; Mondal et al., 2014). Sporadically occurring BL is uncommon and rarely encountered in Western countries. Different lymphomas are associated differently with age. In the main, lymphoma incidence increases with age, but there are exceptions. Hodgkin’s lymphoma has a peak incidence in early adulthood and prevalence is comparatively higher than in other age groups (Sherief et al., 2015; Smith et al., 2015). T-cell lymphomas in Asia also show an increased incidence in childhood (Armitage, 2015). Also, there is a gender difference, as males have an increased risk of lymphomas overall and prevalence is higher in all age groups (Smith et al., 2015).

2.1.3 Treatment

Chemotherapeutic drugs have a major role in the treatment of lymphomas, unlike most solid neoplasms. Permanent remission can be achieved with modern chemotherapeutic drugs in many lymphomas, but not in all. The first cytostatic drugs developed were mustard gas-based. During World War I mustard gas was found to be a suppressor of haematopoiesis (Krumbhaar, 1919). In December of 1942 the first attempts at intravenous nitrogen mustard therapy were recorded. This patient series included a few patients suffering from HL. Although the nitrogen mustard therapy resulted in a short-lived response, this response was often very good and gave hope for further development (Goodman et al., 1946). Chemotherapy regimens are often tailored to different situations. Addition of the monoclonal antibody to CD20, rituximab, has revolutionized B-cell lymphoma treatment. It has increased survival in almost all patient groups. Surgery has less
of a role in treatment than in solid neoplasms and often surgery is only needed for a diagnostic tumour sample. The role of radiotherapy varies between different lymphoma subgroups (Fort, Guet, Colson-Durand, Auzolle, & Belkacemi, 2016).

2.2 Diffuse Large B-cell lymphoma

Diffuse large B-cell lymphoma is the most common lymphoma in Western countries, comprising 30–40% of newly diagnosed cases. It is generally a very aggressive disease and without treatment leads to death – thus intervention is always necessary. Its aetiology is unknown, but a few risk factors have been identified. Epidemiologically its incidence is rising among lymphomas in general and also in relation to other neoplasms. Lymphomas are a very heterogeneous group of diseases in behaviour and in biology and according to the WHO (IV 2016) DLBCLs should be further sub-classified into germinal centre B-cell (GCB) and activated B-cell (ABC) diseases (Alizadeh et al., 2000). There is variation in primary treatment modalities between countries, but new drugs that target distinct genetic variants are in development and clinical trials. In routine practice the only prognostic tool is the International Prognostic Index (IPI), which was developed to stratify the risk of aggressive B-cell lymphomas (A predictive model for aggressive non-hodgkin's lymphoma. the international non-hodgkin's lymphoma prognostic factors project.1993).

2.2.1 Aetiology and epidemiology

Currently the specific aetiology of DLBCL is unknown. There has been increasing evidence of specific precursor mutations that occur in normal B-cells, e.g. progressive transformation of germinal centre (Bouron-Dal Soglio et al., 2008) and transient Bcl-6 expression (M. R. Green et al., 2014). These mutations grant clear survival advantages by altering apoptosis, cell cycle and repair mechanisms in the cell. Indolent lymphomas, e.g. FL and HL can become transformed into more aggressive DLBCL and it has been suggested that \( P53 \) mutations and MYC alterations play a part in the process (Geyer et al., 2015; Pasqualucci L et al., 2014; Schmitz et al., 2005). There is also evidence of a connection between various chronic diseases and DLBCL. In a Japanese population-based cohort study serum HBV positivity was associated with DLBCL at a hazard ratio of 7.22 (95% CI 2.34–22.29) (Abe et al., 2015). A connection between red meat intake and
DLBCL has been established, with a risk ratio of 1.20 (95% CI 1.04–2.37, \( p = 0.01 \)) (Fallahzadeh et al., 2014).

DLBCL is the most common lymphoma in most countries. It comprises about 30 to 40% of all newly diagnosed lymphomas. The average age at diagnosis is about 70. A sharp increase in incidence is seen after the age of 50, as with other neoplasms. Differences in overall survival were noted in a large 40-year surveillance project, between ethnicities, gender and age. In the USA Caucasians showed better survival compared with ethnic minorities, e.g. Asians, Hispanics and African Americans. As regards gender, males seemed to have worse prognosis. Old age was associated with worse treatment outcome (Crozier et al., 2015).

### 2.2.2 Subtypes

Several subtypes can be identified in DLBCL: EBV-positive DLBCL (Ok, Papathomas, Medeiros, & Young, 2013), primary intravascular large B-cell lymphoma (IVLBCL) (Y. Chen et al., 2014), primary cutaneous DLBCL, leg-type (Wilcox, 2015), primary testicular DLBCL (PTL) (L. Deng et al., 2015), primary central nervous system (CNS) DLBCL (PCNSL) (C. Chang, Lin, Cheng, Medeiros, & Chang, 2015), T-cell/histiocyte rich large B-cell lymphoma (T/HRLBCL) (Tousseyn & De Wolf-Peeters, 2011), DLBCL with plasma cell immunophenotype and DLBCL not otherwise specified (NOS) (Menon, Pittaluga, & Jaffe, 2012). Several lymphomas have a close connection with DLBCL: B-cell lymphoma unclassifiable, with features intermediate between BL and DLBCL (B-UNC/BL/DLBCL), primary mediastinal B-cell lymphoma (PMBL) (Steidl & Gascoyne, 2011), and B-cell lymphoma unclassifiable, intermediate between HL and DLBCL (B-UNC/HL/DLBCL).

<table>
<thead>
<tr>
<th>Table 1. DLBCL subtypes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Subtype</strong></td>
</tr>
<tr>
<td>DLBCL, NOS</td>
</tr>
<tr>
<td>PTL</td>
</tr>
<tr>
<td>DLBCL, EBV positive</td>
</tr>
<tr>
<td>Primary cutaneous DLBCL</td>
</tr>
<tr>
<td>PCNSL</td>
</tr>
<tr>
<td>IVLBCL</td>
</tr>
<tr>
<td>T/HRLBCL</td>
</tr>
<tr>
<td>DLBCL with plasma cell immunophenotype</td>
</tr>
</tbody>
</table>
The common features of DLBCL subtypes are presented in the Table 1. The diagnostic criteria of EBV-positive DLBCL of the elderly are age over 50, no immune deficiencies and detectable EBV positivity in neoplastic cells. This entity is more aggressive than the EBV-negative variant and poor survival has been reported, with median overall survival of nine months (C. G. Song et al., 2015). There has been some discussion over whether or not the age limit should be removed from classification, and designation of EBV-positive DLBCL as an independent variant of DLBCL, since prognosis is the same between age groups of EBV-positive DLBCL cases and also because of biological similarities between diseases. In the revised WHO IV 2016 classification age limitation was removed from this entity (T. X. Lu, Liang et al., 2015; Swerdlow et al., 2016). IVLBCL is a rare entity with varying symptoms and it is hard to diagnose, thus often being diagnosed post mortem. It is characterized by large B-cell lymphoma in small blood vessels. It can cause a multitude of symptoms and very rarely has any lymph node involvement. It can be diagnosed with blood vessel biopsy. Two forms of IVLBCL can be identified through clinical symptoms, i.e. a Western and an Asian variety. In the Western form, common places of manifestation are the skin and the CNS. In the Asian form symptoms include hepatosplenomegaly, pancytopenia, multiorgan failure and haemophagocytotic syndrome. With conventional treatment survival is often poor (Kitanaka et al., 2009). Primary cutaneous DLBCL is often diagnosed in elderly persons. Its preferred location is the legs but it can be found elsewhere on the skin. Primary cutaneous DLBCL is among the most aggressive of skin lymphomas. Overall 5-year survival is considered to be about 50%. Treatment modalities are often the same as in systemic DLBCL (Grange et al., 2014).

Primary testicular lymphoma is most known for its frequent relapses, even though complete remission may have been achieved. Common places of relapse are the CNS and the contralateral testicle. Common treatment involves anthracycline-based chemotherapy combined with rituximab, i.e. R-CHOP, CNS prophylaxis and radiotherapy to the contralateral testicle. With intensive therapy 10-year survival is 84% (Vitolo et al., 2011). PCNSL is classified as lymphoma in the intracerebral space, spinal cord, spinal fluid or intraocular space. It is considered to have similarities with PTL. Survival is often poor as a result of poor penetration of chemotherapeutics through the blood brain barrier, but high intensity therapeutics combined with autologous stem cell transplantation have given promising results. With autologous stem cell transplantation 3-year overall
survival has been reported to be 85.2%, compared with 35.2% without it (Madle et al., 2015), but survival varies (Kreher et al., 2015).

T/HRLBCL is connected to nodular lymphocyte-predominant HL (NLPHL), because these diseases are often diagnosed in the same patient at different times and anatomical locations. Also, commonalities have been shown at the genomic level (Hartmann et al., 2015). These neoplasms are often differentiated through immunohistochemistry, but flow cytometry can also be used (Wu, Thomas, & Fromm, 2016). Diagnosis is important due to the fact that T/HRLBCL and NLPHL are treated differently, i.e. T/HRLBCL should be treated as stage-matched DLBCL and NLPHL should treated as standard HL (Abramson, 2006). Plasmablastic DLBCL is characterized by increased expression of plasma cell markers CD38/CD138 combined with weak B-cell and MUM-1 positivity. Several different types of plasmablastic DLBCL can be identified and are often related to different viruses, e.g. HHV-8 to primary effusion lymphoma and plasmablastic lymphoma to HIV. Plasmablastic DLBCL is also related to compromised immune defence, e.g. old age and iatrogenic conditions (Castillo, Bibas, & Miranda, 2015; Rudzki et al., 2005).

PMBL is characterized by enlargement of mediastinal lymph nodes and is generally a disease seen in women between the third and fourth decades of their lives. It is considered to be less aggressive than DLBCL NOS. It is very responsive to treatment and treatment modalities are often the same as with DLBCL. The role of consolidating radiotherapy has been questioned because of long-term consequences (Giri et al., 2015). B-UNC/HL/DLBCL is a lymphoma closely related to PMBL, with biological and clinical similarities. Unlike PMBL it is diagnosed most often in men, but in the same age group as PMBL.

2.2.3 Biological variance

DLBCL can be divided into three groups using gene expression profiling (GEP); the GCB type, ABC type and an unclassifiable third type (Alizadeh et al., 2000; Hans CP et al., 2004). This division is based on comparison of GEP profiles of DLBCL cells and normal germinal centre cells or activated blood B-cells. There is a hypothesis that the cell of origin can explain these genetic differences. The GCB type is thought to originate from germinal centre naive B-cells and the ABC type is believed to be originally an activated B-cell in the periphery outside lymph nodes. In the ABC type there is often constitutive activation of nuclear factor kappa-light-chain-enhancer of B-cells (NFκB) through the B-cell receptor
pathway. NFκB is an important transcription factor that governs DNA transcription and cytokine production; thus it affects cell proliferation and cell survival (Turturro, 2015). It has been hypothesised that even in GCB cases, activation of NFκB plays a role in survival, with worse survival for those with activation (Odqvist et al., 2014). Several different genes have been shown to be more expressed in the GCB type, e.g. Bcl-6 and CD10 (Alizadeh et al., 2000).

Unfortunately, GEP classification is currently a rarely used method as a result of technical difficulty and financial restraints. There is evidence that those with the ABC type have worse prognosis as far as standard kinds of immunochemotherapy are concerned (Nyman, Jerkeman, Karjalainen-Lindsberg, Banham, & Leppa, 2009). The exact nature of this difference is currently unknown, but NFκB has been suggested to be the cause of this difference (Turturro, 2015). Through these GEP differences Hans et al. 2003 created an immunohistochemical method of separating GCB and non-GCB types. This is a commonly used method in clinical practice. The non-GCB type includes ABC and the third unclassifiable type. Hans’s algorithm is shown in Figure 1 and it is based on differential expression profiles in immunohistochemistry between types. The cut-off value for positive expression of CD10, BCL6 and MUM-1 is 30% (Hans et al., 2003). Other immunohistochemical methods have been developed, such as those described by Choi et al. (Choi et al., 2009) and Visco-Young et al. (Visco et al., 2012). Although the standard detection method in the revised WHO IV 2016 lymphoma classification is IHC, there is some evidence that the above-mentioned classification systems and also other immunohistochemical classifications cannot always reveal significant subgroups, when compared with GEP (Coutinho et al., 2013; Gutierrez-Garcia et al., 2011; Nyman et al., 2007; Read et al., 2014). GEP is still considered to be the gold standard in GCB and ABC classification, but new methods are emerging. GCB and ABC sub-typing does not affect treatment modalities in current practice, but there are new emerging drugs that are designed to target genetic differences.

DLBCL can also be classified into different morphological variants; the centroblastic, anaplastic and immunoblastic subtypes. The morphological classification system has been proven by Ott et al. (2010) to be prognostically relevant as regards rituximab, but unfortunately due to low reproducibility, i.e. classification variation between pathologists, it is in limited use in routine practice. The centroblastic variant in studies involving both GEP and morphological sub-typing seems mostly to comprise the GCB subtype, and the immunoblastic variant, the ABC subtype. This may in part explain the prognostic difference (K.
Ohshima et al., 1999; Ott et al., 2010a). Several other immunohistochemical and biological classification systems have also been suggested, with the intent of combining classification and prognosis (Alizadeh et al., 2000; Choi et al., 2009; Deeb, D'Souza, Cox, Schmidt-Supprian, & Mann, 2012; Deeb et al., 2015; Dybkaer et al., 2015; J. S. Hall et al., 2015; Hans et al., 2003; S. Hu et al., 2013; Iqbal et al., 2015; T. X. Lu et al., 2015; Mareschal et al., 2015; Ott et al., 2010b; Scott et al., 2015; Q. Xu et al., 2015). A few of these are presented in simplified form in Table 2.

![Diagram showing Hans's algorithm for GCB/non-GCB subtypes]

*Fig. 1. Hans's algorithm for GCB/non-GCB subtypes*
Table 2. DLBCL classification and prognostic factors

<table>
<thead>
<tr>
<th>Study</th>
<th>Method</th>
<th>Classification</th>
<th>Prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alizadeh AA et al. 2000</td>
<td>GEP</td>
<td>GCB/ABC</td>
<td>Poor for ABC</td>
</tr>
<tr>
<td>Choi WW et al. 2009</td>
<td>Immunohistochemistry (IHC)</td>
<td>GCB/non-GCB</td>
<td>Poor for non-GCB</td>
</tr>
<tr>
<td>Deeb SJ et al. 2012</td>
<td>Quantitative mass spectrometry</td>
<td>Protein based, GCB/ABC</td>
<td>Unknown</td>
</tr>
<tr>
<td>Deeb SJ et al. 2015</td>
<td>B-cell-associated gene signatures</td>
<td>GCB: centrocyte and centroblast</td>
<td>Poor for centroblast</td>
</tr>
<tr>
<td>Hall JS et al. 2015</td>
<td>Branched DNA signal amplification assay</td>
<td>GCB/ABC</td>
<td>Unknown</td>
</tr>
<tr>
<td>Hans CP et al. 2003</td>
<td>IHC</td>
<td>GCB/non-GCB</td>
<td>Poor for non-GCB</td>
</tr>
<tr>
<td>Hu S et al. 2013</td>
<td>IHC+FISH</td>
<td>With/without coexpression of Bcl-2 and MYC</td>
<td>Poor for Bcl-2 and MYC</td>
</tr>
<tr>
<td>Iqbal J et al. 2015</td>
<td>MicroRNA expression</td>
<td>GCB/ABC</td>
<td>Poor for ABC</td>
</tr>
<tr>
<td>Lu TX et al. 2015</td>
<td>FISH+IHC, copy number aberration (CNA)</td>
<td>No CNA/Bcl-2, MYC or double CAN</td>
<td>Poor for Bcl-2, MYC, double</td>
</tr>
<tr>
<td>Mareschai S et al. 2015</td>
<td>Multiplex ligation-dependent probe amplification</td>
<td>GCB/ABC</td>
<td>Poor for ABC</td>
</tr>
<tr>
<td>Ott G et al. 2010</td>
<td>Histological</td>
<td>Morphological</td>
<td>Poor for immunoblastic</td>
</tr>
<tr>
<td>Scott DW et al. 2015</td>
<td>Digital gene expression</td>
<td>GCB/ABC</td>
<td>Poor for ABC</td>
</tr>
<tr>
<td>Xu Q et al. 2015</td>
<td>LIMD1-MYBL1 Index</td>
<td>GCB/ABC</td>
<td>Poor for ABC</td>
</tr>
</tbody>
</table>

2.2.4 Prognostic tools

The most used prognostic method is the IPI. It was originally developed as a result of a need to improve prognostic methods. Before the IPI the Ann Arbor staging system was used (Carbone, Kaplan, Musshoff, Smithers, & Tubiana, 1971). Ann Arbor classification is divided into four stages; Stage I is local disease in one lymph node region, Stage II is disease spread to several lymph node areas but confined to one side of the diaphragm, Stage III is disease spread to both sides of the diaphragm and Stage IV is diffusely spread disease affecting one or more extra-lymphatic organs. Ann Arbor staging was found to be lacking, as it revealed only some of the aggressive forms of DLBCL. Thus, new prognostic methods were then sought. The IPI was created after comparing over 2000 aggressive B-cell lymphoma patients’ data. The patients had been treated with anthracycline-based therapies, e.g. CHOP. Several risk factors were identified: Ann Arbor stage
III–IV, high lactate dehydrogenase, extra-nodal involvement in more than one location, age over 60 and low performance status, *i.e.* WHO grade 2, 3 or 4. The IPI index was created by assigning one point to each risk factor and defining four risk groups: low risk 0–1, low-intermediate 2, high-intermediate 3 and high 4–5 points. Although in the original IPI, treatment was anthracycline-based, it has proved to be an effective method of risk stratification even with modern treatment, *i.e.* R-CHOP. Survival data is presented in Table 3, before and after rituximab, and although time end points in the two studies vary it gives a good picture of how rituximab has improved treatment outcomes (A predictive model for aggressive non-hodgkin's lymphoma. the international non-hodgkin's lymphoma prognostic factors project.1993; Ziepert et al., 2010). Other prognostic tools have also been developed, such as Glasgow prognostic scores, Revised-IPI and NCCN-IPI, but they have not replaced IPI as the gold standard (Y. Kim et al., 2014; Sehn et al., 2007; Zhou et al., 2014).

**Table 3. IPI survival data before and after inclusion of rituximab**

<table>
<thead>
<tr>
<th>Study</th>
<th>Treatment Method</th>
<th>Risk</th>
<th>Time</th>
<th>Overall survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>The International Non-Hodgkin's Lymphoma Prognostic Factors Project 1993</td>
<td>CHOP IPI</td>
<td>low</td>
<td>5-year</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td></td>
<td>low-intermediate</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>high-intermediate</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>high</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Ziepert et al. 2010</td>
<td>R-CHOP IPI</td>
<td>low</td>
<td>3-year</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>low-intermediate</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>high-intermediate</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>high</td>
<td>59</td>
<td></td>
</tr>
</tbody>
</table>

2.2.5 Treatment

The standard treatment for DLBCL is cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) combined with rituximab (R). However, treatment modalities vary and in some institutes the primary therapy is R-CHOP with etoposide (CHOEP). The methodology and role of CNS prophylaxis remains a contested topic. Currently the exact role of radiotherapy is unclear and it is mainly used as consolidation therapy with bulky disease after chemotherapy (Dabaja et al., 2015; Kwon, Kim, Kim, Kim, & Heo, 2015; Mendes et al., 2015). There have also been studies indicating that different immunohistochemical phenotypes, *i.e.* GCB and ABC, should be treated differently (Nowakowski & Czuczman, 2015).
Treatment of DLBCL has been revolutionised by the addition of rituximab to standard chemotherapeutic regimens (Coiffier et al., 2002; Pfreundschuh et al., 2006). The IPI is used to assess potential progression of the disease and if possible to increase the treatments in high-risk patients (J. H. Park et al., 2014). Addition of rituximab has greatly enhanced the survival of all patients. Three years after treatment remission is usually permanent and recurrence is very rare. If relapse occurs during treatment or soon after primary treatment, high-dose chemotherapeutic regimens come into the picture. Most often this means the use of high-dose chemotherapy combined with autologous stem cell transplantation. If this is unsuccessful allogeneic stem cell transplantation can be used. If these treatments fail and relapse occurs again, survival is often very short. Before rituximab, median survival was three months, and after it, 9.9 months (Nagle et al., 2013). Even though rituximab has improved treatment outcomes there is still a relatively large patient group that eventually succumbs to the disease. Several drugs are in development with the aim of exploiting specific genetics of DLBCL. A few of these are presented in Table 4 (Bartlett et al., 2016; Chiron et al., 2014; Ji et al., 2016; Martin et al., 2016; Younes et al., 2014). Great leaps have been taken in the treatment of DLBCL, but still some patients succumb to the disease.

**Table 4. Emerging DLBCL drugs**

<table>
<thead>
<tr>
<th>Study</th>
<th>Drug</th>
<th>Mechanism of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Younes et al. 2014</td>
<td>Ibrutinib</td>
<td>Bruton-like kinase inhibitor (BTK)</td>
</tr>
<tr>
<td>Chiron et al. 2014</td>
<td>Idelalisib</td>
<td>PI3K inhibitor</td>
</tr>
<tr>
<td>Martin et al. 2016</td>
<td>Lenalidomide</td>
<td>Exact mechanism unknown</td>
</tr>
<tr>
<td>Bartlett et al. 2016</td>
<td>Brentuximab vedotin</td>
<td>CD30 receptor</td>
</tr>
<tr>
<td>Ji et al. 2016</td>
<td>Thalidomide</td>
<td>Exact mechanism unknown</td>
</tr>
</tbody>
</table>

### 2.3 Follicular lymphoma

Follicular lymphoma is the second most common lymphoma in Western countries. It is a very indolent disease – median overall survival is over 10 years and even more so after the inclusion of rituximab in standard treatment. Before rituximab it was believed that roughly 30% of patients succumb to FL, in 30% of cases FL becomes transformed into a more aggressive lymphoma, and 30% of cases die of other causes, e.g. other neoplasms and illnesses. The transformation rate with rituximab is unknown but it is considered to be lower or the same. A watch and wait strategy is a valid procedure in stage III and IV FL without symptoms (El-
Galaly et al., 2015). FL is considered to be an incurable disease in connection with standard treatment modalities, but there have been some reports of long remission with autologous stem cell transplantation combined with high-dose chemotherapy (Arcaini et al., 2015; Berinstein et al., 2015; Klyuchnikov et al., 2015). Death due to FL has become an increasingly unusual event.

2.3.1 Aetiology and epidemiology

The exact aetiology of FL, as with most lymphomas, is largely unclear. FL is the second most common NHL in Western countries. Its incidence is rising due to aging but it has also been hypothesized that some unknown factors increase the likelihood of FL. It has been suggested that lectins, including bacterial lectins, could bind to B-cell receptors and stimulate FL cells, and this could link bacterial infection and the aetiology of lymphoma (Coelho et al., 2010; Schneider et al., 2015). Leptin polymorphism has been shown to influence the risk of FL, with the leptin A19G polymorphism appearing to decrease the risk of FL (H. Y. Lin et al., 2015). Human leukocyte antigen (HLA) gene variants have been discovered to influence the risk of FL (Skibola et al., 2012; Skibola et al., 2014). Several other genetic factors influencing lymphomagenesis may be identified in the future.

2.3.2 Biology

The most common mutation in FL is the t(14,18) chromosome translocation, which is present in about 90% of FL patients. This translocation causes constitutive Bcl-2 activation through placing the Bcl-2 gene next to the immunoglobulin heavy-chain enhancer. This gives a distinct survival advantage due to the fact that Bcl-2 is a highly anti-apoptotic protein. Bcl-2 binds to apoptotic Bax and Bak proteins that control mitochondrial permeability. Mitochondrial permeability is an important phase at the beginning of apoptosis (Correia et al., 2015a). Although this translocation makes B-cells immune to senescence, it is not enough for the initiation of cancer. Several other mutations are required for the development of FL (Strasser, Harris, Bath, & Cory, 1990). This is highlighted by the fact that in lymph nodes resected from the normal population about 2.3% contain B-cells with Bcl-2 translocation. It is hypothesized that only a small proportion of these develop into FL (Henopp, Quintanilla-Martinez, Fend, & Adam, 2011). In lymph nodes taken several months before diagnosis in situ, FL has been detected using Bcl-2 translocation (Morita et al.,
In murine models it has been shown that several entries into the GC area are required before Bcl-2-harbouring B-cells acquire mutations reminiscent of FL (Sungalee et al., 2014). Normal B-cells are unlikely to survive repeated entry into the GC (Swaminathan & Muschen, 2014). It has also been suggested that one of the reasons for the lack of permanent treatment outcome is that B-cell subpopulations can harbour precursor mutations. These clones are less mutated and proliferative and thus could be less susceptible to chemotherapeutic agents, hence explaining the incurable nature of FL (Carlotti et al., 2015; M. R. Green et al., 2013; Okosun et al., 2014a). There is some evidence that FL patients with an increased amount of MYC protein expression (detected by IHC) show a more aggressive clinical course. Cases of FL with FISH-detected MYC translocation are very uncommon and have a very aggressive nature (Ichikawa et al., 2015).

The intratumoral environment is considered to be important as regards FL. Microenvironment heterogeneity has been suggested to influence prognosis and survival in FL (Ame-Thomas & Tarte, 2014). Follicular helper T-cells (TFH cells) with certain characteristics such as IL-4 production aid the development of FL and enhance the survival of lymphoma cells (Ame-Thomas et al., 2015). Precursor mutations can modify T-cell responses to B-cells with a decreased amount of surface antigen presentation. This decreases the amount of CD4 helper cells and CD8 cytotoxic T-cells, which could potentially influence the cellular countermeasures to FL (M. R. Green et al., 2015).

Follicular lymphoma can be classified into different morphological groups by using HE staining. The morphological classification is shown in Table 5. Four grades are identified in this classification: Grade 1, Grade 2, Grade 3A and Grade 3B. This division is carried out by the Bernard cell-counting method. Grade 1 disease has 0 to 5 large non-cleaved follicular centre cells (centroblasts), Grade 2 has 6 to 15 centroblasts and Grade 3 disease has over 15 centroblasts per high-power field. Grade 3 is further divided into 3A and 3B. In Grade 3A small cleaved follicular centre cells (centrocytes) are detected and in Grade 3B centroblasts form solid sheets. FL Grade 3B is considered to be an independent disease entity closely related to DLBCL. Grade 3B has infrequent Bcl-6 and Bcl-2 translocations compared with frequent Bcl-2 translocations in lower-grade FL. In one study, in IHC, FL Grades 1, 2 and 3A were 100% CD10-positive and 100% IRF4/MUM1-negative, and in contrast, in Grade 3B 43% of the cells were CD10-negative and 42% were IRF4/MUM1-positive (Horn et al., 2011). The higher the grade the more genetically similar the clones are to each other.
Table 5. Follicular lymphoma morphological classification

<table>
<thead>
<tr>
<th>Grade</th>
<th>Centroblasts per high powered field</th>
<th>Centrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0 to 5</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>6 to 15</td>
<td>+</td>
</tr>
<tr>
<td>3A</td>
<td>Over 15</td>
<td>+</td>
</tr>
<tr>
<td>3B</td>
<td>Over 15</td>
<td>-</td>
</tr>
</tbody>
</table>

2.3.3 Disease transformation

Transformation is a critical event in the pattern of FL disease. The transformation rate is believed to be 3% per year, but variation between studies exits. In some series the transformation rate in ten years is as low as 10% and in some as high as 60%. A lower risk of transformation has been established in actively treated patients (HR 0.57, CI 95% 0.39–0.83). Several clinical factors have been associated with an increased risk of transformation (Al-Tourah et al., 2008; Conconi et al., 2012; Guirguis et al., 2014; Hirayama et al., 2014; Maeshima et al., 2013; Wagner-Johnston et al., 2015). Transformation before the use of rituximab was almost synonymous to lymphoma-related mortality. Rituximab has greatly improved survival odds in cases of transformed FL. In current practice transformation is an indication for stem-cell transplantation, although there is some evidence that upfront treatment without stem-cell transplantation might have its merits (Gleeson et al., 2016; Villa et al., 2013). FL most often transforms to DLBCL, but other aggressive B-cell lymphomas are also encountered, e.g. Burkitt’s and Hodgkin’s (Gheith, Cornfield, Chen, Singh-Kahlon, & Ahmed, 2014; Hwang, Loong, Chung, & Chim, 2011; Shaver et al., 2014), and transdifferentiation to other cell types has been reported, e.g. histiocytic sarcoma (Mehrotra & Pan, 2015; Stoecker & Wang, 2013). Although transdifferentiation is a very rare event in the natural path of FL, it reflects incredible plasticity. Transformed FL is more likely to be of GC type if classified by cell of origin (COO), but a small proportion (about 10%) of cases of FL are ABC or a third type (Kridel et al., 2015).

If the clinical course of FL changes drastically into more aggressive disease, e.g. disease burden dramatically increases, transformation is suspected. Transformation can be confirmed by biopsy before starting aggressive treatments. There is some evidence that PET-TT can be used to distinguish transformed lymphomas from normal FL, as SUV$_{max}$ is higher in transformed cases (Wondergem et al., 2015), but it cannot replace biopsy.
It is believed that there are several different mechanisms of transformation. Some transformation has been established by genetic analysis to arise from a dominant clone with similar mutations as in the original FL. In some cases, transformed FLs harbor mutations that are believed to be precursor mutations as in the dominant FL clone, but are genetically very different. These genetically different clones might come from earlier less mutated clones or from cancer stem cells (Carlotti et al., 2009; Eide et al., 2010).

It is generally accepted that no single mutation causes transformation, but that transformation is the sum of a multitude of actions. TP53 mutations have been identified potential drivers of transformation. Some investigators have even reported a mutation frequency of 28% in transformed FL, and in de novo FL p53 mutation frequency is believed to be around 5% (Davies et al., 2007). Several microRNAs linked to inactivation of TP53 have also been linked to transformation (Lawrie et al., 2009; Thompson et al., 2016). CDKN2A/B, c-REL and MYC have also been linked to transformation (Okosun et al., 2014b; Rossi, 2015).

Several mutations and other factors that increase the risk of transformation into DLBCL have been identified. Bcl-2 mutations increase the risk of transformation (HR 3.6, 95% CI 2.0–6.2, P<0.0001) and in cases of transformed lymphoma about 53% contain Bcl-2 mutations. Bcl-2 mutation frequency might be indicative of a larger mutational load with increased mutation frequency (Correia et al., 2015b; Matolcsy, Casali, Warnke, & Knowles, 1996). CD30 positivity in de novo FL has been linked to an increased frequency of transformation (Montes-Moreno et al., 2015; Shaver et al., 2014). Helper T-cells with FOXP3 positivity have been linked to an increased risk of transformation. This might be indicative of less interaction between other cell components in the neoplasm (Farinha, Al-Tourah et al., 2010). Vascularization of tumour tissue has been linked to a risk of transformation. It is thought that low microvessel density increases angiogenesis and therefore predisposes the tissue to transformation (Farinha, Kyle et al., 2010).

Several alterations in genes have been associated with an increased risk of transformation. One of these is the embryonic stem cell-specific signature, which shows that transformed FLs are more often already less differentiated than their counterparts at diagnosis (Gentles et al., 2009). Several copy number alterations have been associated with an increased risk of transformation. Duplication of chromosome X in men seems to confer an added risk of transformation (Bouska et al., 2014). Other copy number alterations have been established as marking
2.3.4 Clinics

As in DLBCL, Ann Arbor staging is used to divide FL cases into four stages. Risk analysis in a clinical setting can be done using the Follicular Lymphoma International Prognostic Index (FLIPI), which was developed after the success of the IPI in more aggressive B-cell lymphomas. Prognostic factors were determined in a retrospective analysis of 1795 patient histories. Five risk factors were determined: age over 60, over four affected nodal sites, Ann Arbor stage III–IV, lactate dehydrogenase (LDH) level above normal and haemoglobin level under 120 g/L. Three risk groups were established: low risk, with zero to one risk factor, intermediate risk, with two risk factors, and a high-risk group with three or more risk factors (Solal-Celigny et al., 2004). Although B symptoms are not included in FLIPI, Ann Arbor staging includes them, as in DLBCL.

Rituximab has increased the length of survival in patients with transformed FL and median survival is five years after transformation. A few factors that clinically lower the transformation risk have been identified, e.g. rituximab maintenance treatment (HR 0.67, 95% CI 0.46–0.97) and treatment at diagnosis (HR 0.58, 95% CI 0.46–0.75). Clinical factors that seem to increase the risk of transformation at diagnosis have been discovered: poor WHO performance status over one (HR 2.12, 95% CI 1.27–3.55), more than one extranodal site (HR 1.39, 95% CI 1.07–1.81), elevated LDH (HR 1.57, 95% CI 1.17–2.10) and B symptoms (HR 1.35, 95% CI 1.06–1.72). A point of interest is that transformed and non-transformed FL share clinical factors and prognostic indexes are unable to differentiate those at risk (Wagner-Johnston et al., 2015).

2.3.5 Treatment

Current treatment protocols for FL vary greatly. Modern chemotherapeutics can include R-CHOP, R-bendamustine, single rituximab, and watch and wait. Even radiotherapy can be considered as a valid treatment option for a local disease that is limited to one lymph-node area. Due to the very indolent nature of FL, treatment strategies vary greatly, because the long-term survival risk-reward ratio of treatment must be analysed. Younger patients might tolerate more aggressive strategies, but with no lasting remissions, more often treatment must be carefully
analysed. If treatment strategies are too aggressive the risk of secondary malignancies increases and treatment-related mortality increases. Some clinicians consider FL treatment only when disease burden is too great or symptoms are increasing (El-Galaly et al., 2015).

The most important drug in use is rituximab. It can be used as single-agent treatment and in some centres it is used as maintenance therapy to consolidate treatment results. Long-term follow up shows that maintenance rituximab treatment does not affect disease-specific survival, but might lengthen progression-free survival (Ardesha et al., 2014). Therefore, the added benefit of rituximab maintenance in treatment of FL can be debated. Rituximab has been successfully combined with other treatments (as in DLBCL) such as CHOP and CHOP-like regimens. CHOP treatment is an aggressive treatment option with a risk of treatment-related mortality or adverse effects. Some patients might benefit from R-CHOP treatment, but classification of these patients is lacking.

In the last 10 years bendamustine has become a staple in the treatment of FL and, in general, for indolent B-cell lymphomas. Bendamustine combined with rituximab has less treatment-related toxicity and comparable treatment results, i.e. longer PFS, but similar OS, in relation to more traditional CHOP therapies (Flinn et al., 2014; Rummel et al., 2013). Because of this, R-bendamustine has become a popular treatment choice in many centres. Long-term follow-up data concerning this treatment strategy is still lacking. Even though previously mentioned strategies are effective in treatment, no lasting remission has been achieved. Stem-cell transplantation (autologous and allogeneic) in conjunction with high-dose chemotherapy might offer a cure for non-transformed and transformed FL in selected cases (Heinzelmann et al., 2016; Peters et al., 2011).

In transformed FL, treatment options are as in DLBCL, but more commonly very aggressive treatment strategies are used and autologous and allogeneic stem cell transplantation combined with high-intensity cytostatic regimens can be considered. If primary treatment is successful, the DLBCL component of the disease is cured, but these aggressive treatments do not always alter the relapse and remission cycle of FL. If the transformed component of FL relapses, survival is often very poor, as in relapsed de novo DLBCL (Montoto, 2015).

New drugs for FL are currently under development, but due to the indolent nature of the disease these are reserved for special situations. Although treatment strategies have evolved and made FL into a very indolent disease, complete and durable remission is still distant.
2.4 Double-hit lymphomas

Double hit (DH) lymphomas are thought to represent the most aggressive variety of lymphomas. The revised WHO IV 2016 classification added DH lymphomas as a new class of high-grade B-cell lymphomas (Swerdlow et al., 2016). Classified as lymphomas with MYC translocation and Bcl-2 or Bcl-6 rearrangements, these changes can be identified using fluorescence in situ hybridisation (FISH) (Swerdlow, 2014), but not all significant rearrangements can be discovered this way (Ryan et al., 2015). Clinically, DH lymphomas show aggressive behaviour and poor prognosis and more often than not lead to unsuccessful first-line therapy and to more aggressive treatment modalities in younger patients, i.e. high-dose therapy or dose-escalating therapy with or without allogeneic or autologous stem cell transplantation (Howlett et al., 2015). The role of first-line autologous stem cell therapy is unknown. These lymphomas were in several different categories in earlier classification and considered to be as several different types. DH lymphomas can exhibit behaviour and characteristics typical of intermediate DLBCL and BL (Pillai, Sathanoori, Van Oss, & Swerdlow, 2013; Yoshida et al., 2015).

2.5 Oxidative stress

Oxidative stress is an imbalance between reactive oxygen species (ROS) and counteractive elements, e.g. detoxifying and damage-repairing elements. Oxidative stress is present in all cells and without dampening mechanisms, harmful action causes apoptosis and cell death. Therefore, cells have adapted to this inevitable phenomenon via many different mechanisms. As cells became adapted to oxidative stress, many important functions in cellular biology arose, e.g. second messenger and sensory functions (D'Autreaux & Toledano, 2007). Even though cells have adapted, heightened oxidative stress and dysregulation of oxidative stress controllers has been linked to many chronic illnesses including cancer, cardiovascular disease, diabetes and autoimmune diseases (Beyazit, Kocak, Tanoglu, & Kekilli, 2015; Di Minno et al., 2016; Dong et al., 2016; Hecht et al., 2016; Jafari et al., 2016; Kienhofer, Boeltz, & Hoffmann, 2016; Kitano et al., 2016; T. Liu et al., 2015; Malaguti et al., 2014; Martin-Subero, Anderson, Kanchanatawan, Berk, & Maes, 2016; Metwalley, Farghaly, Saad, & Othman, 2016; Radbruch et al., 2016; Salazar-Ramiro et al., 2016; A. A. Shah, Dey-Rao,

Oxidative stress is tightly linked to ROS, as most of the reactive compounds are oxygen radicals. Some examples of these are hydroxide (OH⁻) and hydrogen peroxide (H₂O₂). ROS are produced by endogenous sources, e.g. in normal mitochondrial function as a by-product of energy production, and by exogenously formed chemicals and UV radiation (Mauthe, Cook, Coffing, & Baird, 1995; Shen, Ong, Lee, & Shi, 1995). In normal physiological states the formation of these compounds is tightly controlled and regulated. If scavenging enzymes function improperly or the production of ROS is uncontrolled, they cause damage to lipids, proteins and also DNA. Thus, oxidative stress may trigger mutations and disturb protein functions (Klaunig, Kamendulis, & Hocevar, 2010).

Reactive oxygen species are brought about by electron shortage. This electron shortage makes the compounds highly reactive, with a short life of microseconds. These reactions reduce the reactive compound and oxidize another compound. Often this means reduced or a complete halt to function of a protein. Cells have many proteins and enzymes which counteract this change by reducing the oxidized protein back to normal function and they also have other counteractive compounds. These proteins are integral in the normal function of the cell. Without counteracting mechanisms damage accumulates and leads to uncontrolled cell death. Even reduced function of mitigating enzymes sensitizes the cells to carcinogenesis and apoptosis (Castaldo, Freitas, Conchinha, & Madureira, 2016).

The antioxidant system in cells is diverse and highly effective in neutralizing oxidative stress and ROS. There are many overlapping oxidative protective systems, but also some antioxidants that are specifically localized in different parts of the cell. Some of the most important of these antioxidants are glutamate-cysteine ligase (GCL), peroxiredoxins (Prx), thioredoxins (Trx) and members of the superoxide dismutase (SOD) family. Table 6 presents studies of oxidative stress and lymphomas (Bur et al., 2014; Kuusisto et al., 2015; C. Li et al., 2012; Pasanen et al., 2012; Tome et al., 2005).
Table 6. Oxidative stress enzymes in lymphoma

<table>
<thead>
<tr>
<th>Marker</th>
<th>Function</th>
<th>Study</th>
<th>In lymphoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-OHdG</td>
<td>Marker of oxidative stress to DNA</td>
<td>Bur H et al 2014</td>
<td>High levels associated to poor survival in Hodgkin lymphoma</td>
</tr>
<tr>
<td>Nitrotyrosine</td>
<td>Marker of oxidative stress to protein</td>
<td>Bur H et al 2014</td>
<td>High levels associated to poor survival in Hodgkin lymphoma</td>
</tr>
<tr>
<td>Nitrotyrosine</td>
<td>Marker of oxidative stress to protein</td>
<td>Pasanen AK et al 2012</td>
<td>High levels associated to poor survival in DLBCL</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Mitochondrial redox enzyme</td>
<td>Bur H et al 2014</td>
<td>High levels associated to poor survival in Hodgkin lymphoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tome ME et al 2005</td>
<td>Low levels associated with poor survival in DLBCL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pasanen AK et al 2012</td>
<td>No prognostic value in DLBCL</td>
</tr>
<tr>
<td>Peroxiredoxins</td>
<td>Redox enzyme</td>
<td>Kuusisto ME et al 2015</td>
<td>High levels associated to poor survival</td>
</tr>
<tr>
<td>Trx</td>
<td>Redox enzyme</td>
<td>Li C et al 2012</td>
<td>High levels associated with poor survival in DLBCL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tome ME et al 2005</td>
<td>High levels associated with poor survival in DLBCL</td>
</tr>
<tr>
<td>GCL</td>
<td>Redox enzyme</td>
<td>Tome ME et al 2005</td>
<td>High levels associate with poor survival</td>
</tr>
</tbody>
</table>

2.5.1 Oxidative stress markers

Owing to the short half-life of ROS, it is difficult to observe them directly. Fortunately, surrogate markers have been developed. These markers are often residues of ROS function. One of these is 8-hydroxy-2'-deoxyguanosine (8-OHdG), which has been used to evaluate the potential carcinogenic nature of many environmental factors. It has also been used to study neoplastic tissues and in some has been established as a risk factor of more aggressive disease (Valavanidis, Vlachogianni, & Fiotakis, 2009). Another oxidative stress marker is...
nitrotyrosine, which is a tyrosine residue that has been removed from a protein structure as a result of oxidation. Tyrosine is modified in many physiological and pathological processes. Many experiments carried out in vitro have shown that nitrotyrosine may have many functions and this has created a lot of speculation as to its function in vivo (Ahsan, 2013; De Sanctis et al., 2014).

8-OHdG is thought to be an indicator and one of the most common products of oxidative DNA damage. High energy and close proximity are required to damage the double-helix structure of DNA (Pryor, 1988). Of the four DNA helix nucleobases, guanine has the lowest ionization potential, which makes it the most easily oxidized. Interaction between a hydroxyl radical and the double bond at position C-8 leads to the formation of 8-OHdG. It is the most abundant DNA-damage product in humans (Yin et al., 1995). To measure ROS-caused DNA damage noninvasively, urinary testing of 8-OHdG was developed. The first studies showed increased amounts of 8-OHdG as a result of exercise and tobacco smoke (Chiou et al., 2003; Loft, Fischer-Nielsen, Jeding, Vistisen, & Poulsen, 1993). Later, increased amounts of 8-OHdG in urine were linked to several chronic illnesses, e.g. cardiovascular disease (Di Minno et al., 2016; Martinet, Knaapen, De Meyer, Herman, & Kockx, 2002), diabetes (Hinokio et al., 2002; Negishi et al., 2001; Shin et al., 2001) and neoplasms (Fokinskii, Kotzbach, Szymanki, & Olinksi, 2000). Several environmental factors have also been connected to higher levels of 8-OHdG, e.g. tobacco smoke, and its particulate matter has been connected to higher levels of 8-OHdG in cells in general, and particularly in the lungs (Leanderson & Tagesson, 1990; Xi, Chao, Yang, Zhang, & Zhang, 2005). These results have given 8-OHdG a reputation as a biomarker that is reliable for the evaluation of oxidative stress (Prabhulkar & Li, 2010).

Concerning neoplasms, in one study large uterine myomas were found to be associated with an elevated urinary 8-OHdG content (Fokinskii et al., 2000). Women with breast cancer have shown higher levels of 8-OHdG in blood than controls (Djuric et al., 1996). Similar results have been seen in tissue samples of breast cancer (A. Matsui et al., 2000). In hepatocellular cancer, a higher content of 8-OHdG was detected than in controls (Kitada et al., 2001). In bladder and prostate cancer, higher levels have also been established (Chiou et al., 2003). Urinary levels of 8-OHdG have been analysed in haematological disorders and those patients with higher stage and IPI scores had higher levels (Honda, Yamada, Tomonaga, Ichinose, & Kamihira, 2000). Immunohistochemical analysis of 8-OHdG has been performed in selected lymphomas (Bur et al., 2014; Pasanen et al., 2012). In breast cancer, 8-OHdG expression was an independent prognostic
factor (Sova, Jukkola-Vuorinen, Puistola, Kauppila, & Karihtala, 2010). In urinary bladder carcinoma, 8-OHdG expression was associated with poor survival (Soini et al., 2011). In endometroid-type epithelial ovarian cancer, 8-OHdG was associated with chemoresistance (Pylvas-Eerola, Karihtala, & Puistola, 2015) and poor prognosis (Pylvas, Puistola, Laatio, Kauppila, & Karihtala, 2011).

Nitrotyrosine is partly created by the function of myeloperoxidases (MPOs) (Klebanoff, 2005), which have an important microbicidal role in phagocytes. MPO has also been linked to several diseases. Nitrotyrosine is also created by chronic inflammation brought about by inducible nitric oxide synthase (iNOS). Chronic inflammation is a carcinogenic event (T. Deng, Lyon, Bergin, Caligiuri, & Hsueh, 2016; Korniluk, Koper, Kemona, & Dymicka-Piekarska, 2017). Increased expression of nitrotyrosine in immunohistochemistry has been linked to breast cancer (Karihtala, Kinnula, & Soini, 2004). Similar results were not observed in blood analysis, as nitrotyrosine levels were similar in controls and in cases of carcinoma (Jin, Daly, Marks, & Zangar, 2013). Expression of iNOS and nitrotyrosine has also been linked to colon cancer (Gochman et al., 2012) and other cancers (Crowell, Steele, Sigman, & Fay, 2003; H. Ohshima, Tazawa, Sylla, & Sawa, 2005). Some reports have indicated that those tumours with an elevated level of nitric oxide are more resistant to chemotherapeutic drugs and are more aggressive in general. Nitric oxide has been linked to advanced stage, poor survival and mesenchymal to epithelial transition in lung cancer (Luanpitpong & Chanvorachote, 2015; Powan & Chanvorachote, 2014). Cisplatin resistance in lung cancer cells has been connected to nitric oxide (Matsunaga et al., 2014). Nitric oxide levels are also associated with melanoma cell resistance to cisplatin (Godoy, Anderson, Chowdhury, Trudel, & Wogan, 2012). iNOS inhibition in some studies has been considered to be a potential mechanism of action of some experimental drugs (Ito, Ando, & Seishima, 2015).

2.5.2 Manganese superoxide dismutase

The SOD family is group of antioxidant molecules that have a redox-active metal. SODs combine two superoxide molecules into oxygen and hydrogen peroxide. Metal is an important part in the stabilization process of highly reactive compounds. SODs probably developed over 2.4 billion years ago as a result of photosynthesis of cyanobacteria. Several different SODs have evolved, e.g. MnSOD and FeSODs, Cu/ZnSODs and NiSODs. SODs have different localizations in different organisms. In humans MnSOD is solely a mitochondrial
enzyme, but in yeast and plant cells other cellular organs also contain it (Sheng et al., 2014). In humans SODs are MnSOD and Cu/ZnSODs.

MnSOD was discovered in *E. coli* by Keele *et al.* in 1970 (Keele, McCord, & Fridovich, 1970). In human cells MnSOD is exclusively found in mitochondrial matrix. The importance of MnSOD is immense, as mouse embryos with homozygous inactivation die within the first ten days (Y. Li *et al.*, 1995). Mice with heterozygous inactivation have several complications, *e.g.* degeneration of neurons and severe anaemia. Also, mice with heterozygous MnSOD deficiency die within three weeks of birth (Lebovitz *et al.*, 1996). MnSOD is considered to be the only enzyme in the SOD family that is essential for survival in an aerobic environment, as seen in both experiments with mice.

Several transcription factors have been connected to regulation of MnSOD gene expression, *e.g.* NFκB, p53 and activating protein 1. NFκB binds to a consensus motif in the MnSOD gene. Promoter and enhancer regions of the MnSOD gene contain NFκB-binding elements (Jones, Ping, & Boss, 1997; Y. Xu *et al.*, 1999). Several factors induce MnSOD activation through NFκB, *e.g.* ionizing radiation (Akashi *et al.*, 1995; Eastgate *et al.*, 1993) and inflammatory cytokines (Visner, Dougall, Wilson, Burr, & Nick, 1990; Wong & Goeddel, 1988). It has been suggested that p53 regulates the transcription of MnSOD under oxidative stress, but a specific binding site for p53 is still unknown (Drane, Bravard, Bouvard, & May, 2001; Hussain *et al.*, 2004; Kinscherf *et al.*, 1998). Nrf2 has also been suggested to induce antioxidant enzymes including MnSOD (Kwak, Itoh, Yamamoto, Sutter, & Kensler, 2001).

MnSOD gene polymorphism has been associated with an increased risk of breast cancer in some studies (OR 1.87, CI 95% 1.09–3.19 (Cox, Tamimi, & Hunter, 2006) and OR 2.5, 95% CI 1.39–4.54 (Bica *et al.*, 2009)), but an overall meta-analysis showed that no significant association is present (Qiu *et al.*, 2010). Although a link between polymorphism and several other cancers has been suggested, *e.g.* lung (Zejinovic, Akev, Yilmaz, & Isbir, 2009) and bladder cancer (Hung *et al.*, 2004), no large confirmatory meta-analysis has been published. Hence, further studies to elucidate such a connection must be conducted.

Studies of MnSOD expression in tumours have led to partly contradictory results. In some cases, MnSOD levels are lower in tumour tissues and some studies even show that forced over-expression leads to a normal growth pattern, as in an experimental model with a human fibroblast cell line (Yan, Oberley, Zhong, & St. Clair, 1996). Some evidence has been presented that elevated expression is connected to cancer. In gastric (Izutani *et al.*, 1998), colorectal
(Janssen et al., 1998), mesothelial (Kahlos et al., 1998) and breast cancer (Portakal et al., 2000), higher levels of MnSOD have been detected and in some cases linked to poor survival. In contrast, some studies have revealed decreased expression when compared with controls, e.g. studies on colorectal (Van Driel et al., 1997) and lung cancer (Coursin, Cihla, Sempf, Oberley, & Oberley, 1996).

The importance of MnSOD in normal cellular function is undisputed. Its relationship to different neoplasms is currently unclear. Further studies are needed to elucidate the different circumstances in which higher concentrations of MnSOD are harmful as regards prognosis and when lower concentrations are harmful.

2.5.3 Glutathione-related antioxidant defence

Glutathione exits in cells in thiol-reduced (GSH) and disulphide-oxidized (GSSG) isoforms. GSH is the most dominant isoform, and only 2% is GSSG (Akerboom, Bilzen, & Sies, 1982). GSH is localised in different areas of the cell. The largest amount of GSH has been observed in the cytoplasm. GSH is the most important and abundant non-protein that counteracts oxidative stress. It is transformed into GSSG by GSH peroxidase (GPx)-catalysed reactions (S. C. Lu, 1999). These reactions reduce hydrogen peroxide and also other molecules. At the same time GSH is oxidized into GSSG. GSSG can be returned to its functional form GSH by NADPH.

An increased amount of GSH is associated with a proliferative response in many normal and malignant cell types (Huang et al., 2001; J. P. Shaw & Chou, 1986). Levels of GSH increase in hepatocytes when cells switch from G0 to G1 (S. C. Lu & Ge, 1992). In metastatic melanoma cells increased levels of GSH have been related to growth (Carretero et al., 1999). There has been some speculation that GSH localisation in the nucleus during proliferation might change some histone activity. GSH has been considered to play an important role in DNA synthesis. DNA synthesis requires thioredoxins for the activity of the rate-limiting enzyme ribonucleotide reductase. Thioredoxin needs GSH reduction to function (Diaz Vivancos, Wolff, Markovic, Pallardo, & Foyer, 2010). GSH is known to regulate cell death, as its levels affect the levels of caspases and signalling molecules of apoptosis (Ballatori et al., 2009). GSH levels fall during normal apoptosis, but total GSH depletion leads to necrotic cell death (A. G. Hall, 1999). Decreases of GSH levels are due to reduced activity of GCL, ROS and GSH efflux (Franklin et al., 2002).
GSH levels in different cancers have been extensively studied and in some studies high levels of GSH have been associated with drug resistance and poor prognosis (Gamcsik, Kasibhatla, Teeter, & Colvin, 2012). In breast cancer several studies on GSH immunohistochemistry have revealed a link between higher stage and higher grade (Coban et al., 1998; Kumaraguruparan, Kabalimoothy, & Nagini, 2005), but contradictory data has also been presented (Perquin et al., 2001). No association between survival and GSH in breast cancer has been detected (Woolston et al., 2011). In gastrointestinal cancers, some evidence of an association between higher levels of GSH and poor prognosis have been published (Barranco et al., 2000; Skrzydlewska et al., 2005). The effects of GSH levels are currently unstudied in DLBCL and FL.

GCL is an important rate-limiting enzyme in the synthesis of glutathione. Lower levels of GCL are associated with lower levels of GSH. Drug-resistance of cells and also oxidative stress are connected to GCL activity and levels. GCL levels and the transcriptional activity of GCL rise during oxidative stress. The GCL promoter region has elements for several different oxidative stress transcriptional factors, e.g. NFκB and activator protein-1 (S. C. Lu, 2013). Nrf2 is also thought to activate the GSH system through increasing the transcription of GCL (Chan, Han, & Kan, 2001). GSH and Trx pathways are important in carcinogenesis and also cancer progression. If GCL synthesis is blocked at the onset of cancer, progress halts. If Trx and GSH are inhibited synergistically in cancer cells it leads to cell death (Harris et al., 2015). Some evidence of higher GCL transcription and drug resistance has been presented, but no certainty exists (N. Y. Song et al., 2011; Veas-Perez de Tudela, Delgado-Esteban, Cuende, Bolanos, & Almeida, 2010).

### 2.5.4 Thioredoxins

Thioredoxin is a very important cellular reducing enzyme, which was discovered in the early 1960s. Later, two different isoforms were discovered: Trx1 (Tagaya et al., 1989) and Trx2 (Spyrou, Enmark, Miranda-Vizuete, & Gustafsson, 1997). Trx1 is localised in cytoplasm and Trx2 in mitochondria. Trx functions by donating a hydrogen atom to other reductases and reducing the reductases back to normal function. Thus, Trxs are electron donors for a multitude of enzymes, e.g. Prx and ribonucleotide reductases. Through redox reactions Trx influences many cellular functions, e.g. immunomodulation (Bertini et al., 1999; Nakamura, Nakamura, & Yodoi, 1997), regulation of several transcription factors (Schenk, 42
In regulation of transcriptional factors Trx has been observed to inhibit NFκB and activate activating protein-1, which are both major regulators of the response to oxidative stress (Schenk et al., 1994). Trx over-expression has been shown to inhibit p53-mediated apoptosis in experimental models (Kamoun et al., 2015; Manoharan, Seong, & Ha, 2013).

In aging, the role of ROS has been hotly debated. Theories suggest that the aging process is partly due to ROS. Trx has been studied in relation to aging and the ROS theory. Trx1 over-expression has been shown to increase the median life span in transgenic mice. The median increase in life span was mainly due to better protection from inflammation at an early age. In older mice, this difference diminished due to an increase in the amount of malignancies (Mitsui et al., 2002; Pérez et al., 2011). In Trx1- or Trx2-deficient transgenic mice, homozygous null Trx1 or Trx2 knockout mice do not survive past embryonic stage (M. Matsui et al., 1996; Nonn, Williams, Erickson, & Powis, 2003). Heterozygous transgenic mice had a lifespan similar to that of WT mice, but with a reduced amount of neoplasms. Cells of heterozygous mice were more sensitive to apoptosis (Pérez et al., 2008). Mice with concurrent Trx1 and Trx2 heterozygous knockout had a longer lifespan and mice with concurrent over-expression of Trx1 and Trx2 had a shorter lifespan (Cunningham et al., 2015). Although Trx is essential to normal cell function, some evidence of the harmful role of over-expression has been presented.

In chronic illnesses Trx has been associated with several disorders, but the pathogenic processes behind these associations are still largely unclear. High Trx levels and oxidative stress profiles have an association with insulin resistance in patients with familial combined hyperlipidaemia (Martinez-Hervas et al., 2016). Trx1 levels are associated with the amount of plaque in atherosclerotic patients, with higher levels predicted greater plaque formation (Madrigal-Matute et al., 2015). Insulin resistance has been connected with increased levels of Trx. Insulin resistance seems to play a role in Alzheimer’s disease; thus thioredoxin might affect the pathogenesis of both diseases (Rosales-Corral, Tan, Manchester, & Reiter, 2015). Some evidence has been presented that Trx may have a role in the pathogenesis of several lung disorders. Increasing Trx expression in preclinical models seems to protect against pulmonary tissue injury (J. Xu, Li, Wu, & Xu, 2012).

The roles of Trx in cancer seem to be diverse. As some investigators have noted, increased expression of Trx might sensitize cells to malignant cell
formation. This could be due to inhibition of apoptosis. Trx levels in some cancers are notably higher than in control tissue samples (Mollbrink et al., 2014) and Trx1 serum levels have been suggested as a biomarker in cases of ovarian cancer (B. J. Park, Cha, & Kim, 2014b), mesothelioma (Tabata et al., 2013) and breast cancer (B. J. Park, Cha, & Kim, 2014a). In hepatocellular cancer, increased expression of Trx1 has a connection with cell proliferation and lower expression of Trx2 with increased size (Mollbrink et al., 2014). In small-cell lung cancer, high-level Trx expression is associated with poor prognosis (Kakolyris et al., 2001). In several studies inhibition of Trx has increased apoptosis and sensitized cells to cytostatic drugs. In DLBCL Trx inhibition sensitized cells to doxorubicin (C. Li et al., 2012). In lung cancer the efficacy of carboplatin was increased with dual inhibition of GSH and Trx (Fath, Ahmad, Smith, Spence, & Spitz, 2011). The pathogenesis of neoplasms might be influenced by Trx and some evidence of drug resistance associated with high-level expression of Trx has been shown.

2.5.5 Peroxiredoxins

Peroxiredoxins are family of reductases that utilise cysteine-containing active sites to reduce peroxides in various cellular compartments (Fatma, Kubo, Sharma, Beier, & Singh, 2005; Wood, Poole, & Karpplus, 2003; Wood, Schröder, Harris, & Poole, 2003). Six different isoforms of Prx are present in human cells. Prxs can be further divided into two groups according to the amount of cysteine residues which are redox-active. Prxs I–V have two active cysteine sites and Prx VI has one active cysteine site (Kang, Baines, & Rhee, 1998; Schroder et al., 2000). The amount of cysteine does not seem to affect the role or activity of Prxs (Dayer, Fischer, Eggen, & Lemaire, 2008). As with other antioxidants, when reducing compounds Prxs become oxidized. Prxs I–V are reduced by Trx, and Prx VI by GSH (Poole, Hall, & Nelson, 2011). Recent studies have shed light on the more exact structure and classification of Prxs in various species. In this classification the Prx1 subgroup includes Prxs I–IV. The Prx5 subgroup includes Prx V and the Prx6 subgroup Prx VI (Nelson et al., 2011).

One of the most important functions of Prx is to regulate the intracellular amount of hydrogen peroxide. Some signalling functions of peroxiredoxins have recently been identified. Prx III, which is in mitochondria, is inactivated reversibly by hydrogen peroxide, thus becoming hyperoxidized. In a murine model, it was shown that adrenal steroidogenesis was suppressed by hyperoxidation of Prx III independently of other regulators (Kil et al., 2012). It
has been suggested that Prxs play an important role in apoptosis (Hampton & O'Connor, 2016). Early studies showed that a high amount of Prx delayed apoptosis (Ichimiya, Davis, O'Rourke, Katsumata, & Greene, 1997; P. Zhang et al., 1997) and depletion of Prx III sensitized cells to apoptosis (T. S. Chang et al., 2004; H. Zhang, Go, & Jones, 2007). The Prx III gene is a target of c-MYC and in knockdown studies it was shown that c-MYC function depended partly on Prx III (Wonsey, Zeller, & Dang, 2002). Peroxiredoxins have been shown to be involved in immune responses, as malarial Prx has been shown to react with Toll-like receptor 4 (Furuta et al., 2008). Prx I release is associated with poor prognosis and probable induction of systemic inflammation in cardiogenic shock patients (C. H. Liu et al., 2016). These studies show the important role of hydrogen peroxide regulation and it is highly probable that new functions of Prxs will be discovered in the future.

Increased expression of Prxs has been reported in several cancers. In malignant mesothelioma Prxs I, II, III, V and VI have been found to be highly expressed, but no correlation between clinical factors was established (Kinnula et al., 2002). In another study, in breast cancer Prx I, Prx II and Prx III were highly expressed in comparison with normal tissues, but no relationship between clinical features and Prx was found (Noh et al., 2001). In a later study of breast cancer a connection was found between oestrogen receptor positivity and high-level Prx I expression in patients with improved survival (O'Leary et al., 2014). In breast cancer the expression levels of Prxs III and IV have been associated with better prognosis and that of Prx V with poor prognosis (Karihtala, Mantyniemi, Kang, Kinnula, & Soini, 2003). In lung cancer, compared with controls, high-level expression of Prxs I and III has been reported, but no connection between clinical features was established (J. H. Park et al., 2006). In epithelial ovarian cancer platinum resistance has been connected to high Prx III levels (X. Y. Wang, Wang, & Li, 2013). In prostate cancer Prx III in cellular membranes has been associated with anti-androgen resistance and hydrogen peroxide resistance. Knockdown of Prx III has restored sensitivity to hydrogen peroxide (Whitaker et al., 2013). In oral squamous cell carcinoma, Prx I levels have been found to be higher in cancer tissue than in controls. Prx I has also been linked to epithelial mesenchymal transition, which is important in metastases (W. Niu et al., 2016). In cervical cancer Prx III has been associated with high-level Ki-67 expression and thus with elevated proliferation (J. X. Hu, Gao, & Li, 2013). Prxs may have vital roles in chemoresistance and carcinogenesis. An indication of this carcinogenic potential can be detected, as higher levels of Prxs are present in neoplastic tissues than in
controls. In pancreatic neoplasms loss of Prx expression has been associated with an aggressive phenotype (Isohookana, Haapasaari, Soini, & Karihtala, 2016). In melanoma, dysfunction of Prxs has been shown, with Prx III expression in stromal fibroblasts being associated with poor survival (Hintsala, Soini, Haapasaari, & Karihtala, 2015). In HL, Prx III expression has been associated with advanced stage and Prx V expression with a lower rate of complete response (Bur et al., 2014). Down-regulation of Prx II might play a part in the pathogenesis of endometriosis-associated ovarian cancer, as lower levels than in controls are observed in tissue close to being malignant, with the lowest levels in cancer (Sova et al., 2012). In triple-negative breast cancer, lower levels of Prx IV have been observed than in non-triple-negative breast cancer (Karihtala, Kauppila, Soini, & Jukkola-Vuorinen, 2011). In ovarian carcinogenesis high-level expression of Prxs seems to be an early event (Pylvas, Puistola, Kauppila, Soini, & Karihtala, 2010).

Because of the association between Prx levels and neoplasms, several compounds that target Prxs have been developed. Thiostrepton, a Prx III irreversible inhibitor, and gentian violet, a Trx 2 target compound, seem to have a synergistic effect in malignant mesothelioma in mouse xenografts. The combination lessened tumour burden and showed good tolerability in mammals (Cunniff et al., 2015). In cell xenograft models, Prx I down-regulation by β-elemene increases the radiosensitivity of lung adenocarcinoma (G. Li et al., 2015). A thiol-specific small molecular peptidomimetic, SK053, creates covalent bonds between Prxs, thus irreversibly inactivating them. In BL, this has led to cell cycle arrest and apoptosis (Trzeciecka et al., 2016). These target compounds might have added benefit in several cancers if tolerability is not an issue.

### 2.6 Tumour protein 53

Tumour protein 53 is one of the most recognised molecules in the formation of neoplasms. TP53 mutation is found in up to 50% of all cancers and several indirect inactivation molecules, e.g. MDM2, have been discovered. p53 controls the cell cycle, apoptosis and DNA repair mechanisms. These functions have led p53 to be nicknamed guardian of the genome (Levine, 1997). Other proteins that belong to the same group of transcriptional factors are p63 and p73 (Bourdon, 2007). Recently, studies have shown that some p53 mutant proteins are oncogenic and might be active participants in oncogenic transformation.
2.6.1 Structure

The TP53 gene is located in chromosome region 17p13.1 and it encodes the 393 amino acid-long tumour protein p53. In its functional state p53 is a homo-tetramer. Each of the monomers has an N-terminal transaction region which is divided into two sub-domains, transactivation domain I (TADI) (residues 1–42) and TADII (residues 43–73), a proline-rich domain that has multiple sequences of PXXP (residues 65–97), a DNA-binding domain (residues 102–292), a hinge region with the main nuclear localization signal (residues 300–325), a C-terminal oligomerisation domain (residues 325–356) and a basic region (residues 363–393) (Marcel & Hainaut, 2009; May & May, 1999; Saha, Kar, & Sa, 2015).

The N-terminal transaction domain is vital for contact between p53 and transcriptional co-activators and co-repressors. When MDM2 binds to the N-terminal transaction domain, the form of the secondary structure (Thr18 to Leu26) changes from a coil to a helix (Dawson et al., 2003; Kussie et al., 1996). Simulation studies regarding structural changes in the N-terminal domain with MDM2 interaction have led to the hypothesis that the most important amino acids are Phe19 and Trp23 (Joseph, Madhumalar, Brown, Lane, & Verma, 2010; Lee et al., 2000). The structural change caused by MDM2 stops p53 interaction with DNA and makes the protein sensitive to ubiquitination.

The DNA-binding domain is composed of a traditional loop-sheet-helix motif, and L2 and L3 loops. The traditional loop-sheet-helix motif contains the L1 loop (Phe112–Thr123), beta strands S2 and S2’ and the C-terminal region. The traditional loop-sheet-helix structures bind to the major groove of DNA. The L2 (residues 164–176 and 182–194) and L3 loops (residues 237–250) bind to the minor groove of DNA (Cho, Gorina, Jeffrey, & Pavletich, 1994). L1 and L2 together establish stable DNA-p53 binding. The L2 and L3 loops contain Zn2+ ion and its tetrahedrally held by Cys176, His179, Cys238 and Cys242 (Joergers, Allen, & Fersht, 2004). If loss of Zn2+ occurs the p53 structure is thought to become unstable and easily aggregated (Duan & Nilsson, 2006).

The L1 loop has a very low rate of mutation due to the low amount of amino acids. It is known to have a very disordered secondary structure. It is speculated that this disordered structure is important for DNA binding (Lukman, Lane, & Verma, 2013). Several hotspot mutation areas have been discovered in p53. About 95% of all p53 mutations are located at the DNA-binding domain (Olivier et al., 2002). This high mutation frequency is due to fact that the core DNA-binding
domain has low intrinsic and thermodynamic stability (Khoo, Andreeva, & Fersht, 2009; Pérez Cañadillas et al., 2006).

Several p53 isoforms have been identified that are caused by different translational events. These events come about because TP53 has several alternative splicing regions, several promoters and alternative translation-initiation sites. Most of the protein isoforms retain the DNA-binding domain. The major differences detected are in the N- and C-terminal regions. Isoforms of p53 are thought to be expressed in a tissue-specific manner because some tissues have a higher number of certain isoforms. Expression of isoforms also varies between malignant and normal tissues, as is seen in breast cancer versus normal breast tissue, and the C-terminal-deleted isoform in neuroblastoma (Bourdon et al., 2005; Goldschneider et al., 2006; Marcel & Hainaut, 2009). Isoforms seem to modulate the effect of p53 and have autonomous functional properties (Courtois et al., 2002).

Post-translational modifications are vital in normal cellular function. In the process, different functional groups are added to amino acids. This process changes the function of post-translated protein. p53 is known for several post-translational modifications: phosphorylation of Ser/Thr, ubiquitination, sumoylation and acetylation of Lys residues. These changes have a multitude of effects on p53 function, e.g. they inhibit MDM2 interaction, stabilise/destabilise protein, inhibit apoptosis and cause apoptosis (Saha et al., 2015).

Post-translational modifications are a normal part of p53 function and several have been identified. Phosphorylation of p53 is caused by various mechanisms and in p53 several target areas have been identified. Ser15 and 37 phosphorylation is caused by UV- and γ-radiation and it impairs interaction between MDM2 and p53 (Shieh, Ikeda, Taya, & Prives, 1997; Tibbetts et al., 1999). Phosphorylation of Ser392 is often caused by UV-radiation. This phosphorylation enhances p53 activity (Keller et al., 2001). Phosphorylation of Ser315 and 376 is caused by endoplasmic reticulum stress. This stress inactivates the p53 apoptotic pathway and increases cytoplasmic localization of p53 (Qu et al., 2004). Sumoylation and acetylation have some similar effects in comparison with phosphorylation. Acetylation of Lys320, 373 and 382 causes BAX activation and therefore apoptosis (Yamaguchi et al., 2009). Lys320 acetylation has also been linked to p53-mediated cell-cycle arrest (Sakaguchi et al., 1997). Lys386 sumoylation of p53 increases transcriptional activity (Rodriguez et al., 1999).
2.6.2 Function

p53 has a broad range of different functions which control a wide range of cellular processes, *e.g.* proliferation, growth, DNA repair, cell survival and metabolism. Anticancer roles of p53 include apoptosis, limitation of stem-cell renewal (Aloni-Grinstein, Shetzer, Kaufman, & Rotter, 2014) and restraint of cell migration (Muller, Vousden, & Norman, 2011). p53 has many opposing roles, as it can decrease and increase oxidative stress, drive cell death or help cell survival. The opposing roles and multitude of structural changes make the overall picture of p53 function hard to grasp (Kruiswijk, Labuschagne, & Vousden, 2015).

One of the first identified functions of p53 was induction of apoptosis in neoplastic cells (P. Shaw et al., 1992; Yonish-Rouach et al., 1991). This process is brought about through transcriptional activation of several pro-apoptotic proteins including Bel-2 and p53-upregulated modulator of apoptosis (PUMA) (Chipuk & Green, 2006) and p53 also interacts in cytoplasm with different pro-apoptotic proteins, increasing mitochondrial outer-membrane permeability (Vaseva & Moll, 2009). Mutations of the DNA-binding domain inactivate both the transcriptional and cytoplasmic apoptotic pathways; thus most mutations inactivate apoptotic function (Follis et al., 2014). Many other p53-dependent mechanisms of apoptosis have been identified (Yu & Zhang, 2005). Mechanisms of cell death other than apoptosis have been identified. p53 seems to play an important role in regulated necrosis. In ischaemia p53 accumulates in the mitochondrial matrix and interacts with cyclophilin D, which opens the mitochondrial pores and induces necrosis (Vaseva et al., 2012). Several other forms of necrotic cell death caused by p53 have been reported, *e.g.* podoptosis (Thomasova et al., 2015) and ferroptosis (Jiang et al., 2015).

Cell death is not the only control mechanism of cell fate affected by p53. It can also induce cell cycle arrest temporarily or even permanently if DNA damage is too great or stress continues. Senescence and differentiation are the main mechanisms of permanent inhibition of proliferation. p53-induced senescence is most often caused by disruption of telomere function (Y. Deng, Chan, & Chang, 2008) and oncogene activation (Halazonetis, Gorgoulis, & Bartek, 2008). The main mechanism of p53 senescence is though activation of p21. p21 is a cyclin-dependent kinase inhibitor and it stops the cell cycle in the G1 phase (Brown, Wei, & Sedivy, 1997). Senescence can also be achieved through transcriptional activation of PI3K pathway inhibitors (Coppe et al., 2008). This transcriptional activation is a dual-edged sword, as in some instances it promotes growth and
invasion (Childs, Baker, Kirkland, Campisi, & van Deursen, 2014). Activation of p53 can also prevent cancer cells acquiring stem-cell capabilities and disrupt the plasticity of these malignant cells (Aloni-Grinstein et al., 2014). DNA repair mechanisms of p53 include cell-cycle disruption through p21 and activation of protein poly(ADP-ribose) polymerase 1 (PARP1). PARP1 repairs single-strand breaks of DNA and thus prevents the accumulation of mutations (Wei & Yu, 2016).

Autophagy has been identified as an important function of p53 that limits the rise of premalignant lesions. p53 induces autophagy by way of several target genes, but autophagy also restrains p53 by limiting the activating signals and by p53 degradation (Choudhury, Kolukula, Preet, Albanese, & Avantaggiati, 2013). If autophagy is lost, premalignant lesions are formed, but these lesions do not progress to more malignant cancer without secondary modification, e.g. total loss of p53 (Rao et al., 2014; Rosenfeldt et al., 2013). In a model of pancreatic cancer, loss of autophagy combined with heterozygous loss of p53 function increased premalignant lesion formation, but did not accelerate malignant progress of premalignant lesions (Yang et al., 2014).

Redox regulation has been linked to p53 function. p53 activity is induced by ROS and p53 increases antioxidant defences under normal oxidative stress, but under severe oxidative stress it increases pro-oxidant activation (Maillet & Pervaiz, 2012). p53 has been linked to the maintenance of GSH levels during nutrient starvation, which increases survival of neoplastic cells under harsh conditions (Maddocks et al., 2013). p21 seems to stabilise Nrf2, thus increasing antioxidant capabilities (W. Chen et al., 2009), but p53 suppresses Nrf2-induced transcriptional factors (Faraonio et al., 2006). Senescence and the apoptotic pathway related to p53 are considered to be pro-oxidant events. p53 seems to down-regulate MnSOD levels, thus increasing oxidative stress in severe oxidative stress situations (Drane et al., 2001; Hussain et al., 2004).

It seems that p53 plays an important role in the control of metabolism. This role is closely connected to cell-cycle regulation- and cell death-inducing pathways. p53 can become activated via problems of ribosome biogenesis. In conditions of nutrient deprivation, MDM2 binds to ribosomal proteins, inhibits their normal function and stabilises p53, which leads to senescence (T. H. Kim, Leslie, & Zhang, 2014). When there is a lack of the vital amino acids folate and methionine, p53 induces apoptosis (Hoeferlin, Fekry, Ogretmen, Krupenko, & Krupenko, 2013; Shiraki et al., 2014). p53 can also control the influx of glucose into the cell by reducing the transcription of glucose transporters type 1 and type
2 (C. Zhang et al., 2013). Inhibition of NFκB by p53 reduces the effect of glucose transporter type 4 (Kawauchi, Araki, Tobiume, & Tanaka, 2008). p53 activation lowers the rate of glycolysis and favours mitochondrial energy output and also aids survival in nutrient-starved situations (Kruiswijk et al., 2015).

2.6.3 Mutations

The multitude roles of p53 highlight the importance of this protein. These roles show that changes of p53 function have many different outcomes depending on the situation. Due to the complex nature of its function, it is not surprising that p53 dysfunction can induce cancer formation and even promote the survival of neoplasms (Haupt, Raghu, & Haupt, 2016). Some evidence shows that some mutations harbour a potential for gain of function and mutated p53 blocks the function of normal p53 (Kamp, Wang, & Hwang, 2016).

Early experiments concerning TP53 mutations with animal models were performed by removing the exons coding the DNA-binding domain. Mice with homozygous TP53 deletion were exceedingly sensitive to neoplastic formation, especially sarcomas and T-cell lymphoma. There was an increase in formation of carcinomas, but not to the extent in which it occurs in humans or other mammals. Mice with homozygous deletion fared better than homozygous mutants, but mice with homozygous deletion were more prone to carcinogenesis than wild-type animals. It was also noted that heterozygous mutants had a different distribution of neoplasms than either of the controls (Donehower LA et al., 1992). This raised the question of why the loss of p53 function did not increase the number of carcinomas to the same level as in humans. Use of two murine models answered these questions. First, telomere length in one set of experimental mice was greater than in humans. In a mouse model with short telomeres and p53 aberration the distribution of malignancies was more closely related to those of humans, with more abundance of epithelial cancers. This showed that mouse models could not without further modification simulate human biology (Artandi et al., 2000). The second mouse model depicted a situation in which a murine BALB/c line with a predisposition to mammary carcinomas brought about by radiation was combined with p53 deletion. These mice had a particular predisposition to mammary carcinogenesis, as half of the females died of mammary carcinoma. This model showed the situational effect of p53, as the added insult to mammary cells changed the predisposition to cancer formation (Kuperwasser et al., 2000). These models show that p53 deletion greatly increases the risk of neoplasm formation,
but the situation is also circumstantial and some cells are more sensitive to p53 mutation than others (Kenzelmann Broz & Attardi, 2010).

Although over 1800 different amino acid changes in p53 have been identified (Soussi, 2011), the hotspot mutations, e.g. at residues 175, 245, 248 and 273 account for over 21% of sporadic mutations. Mutations can be coarsely divided into two categories, conformational and DNA contact. Conformational changes can cause the protein to fold into a totally inactive form. DNA contact changes prevent interaction between DNA and p53, but otherwise the protein can function. As conformational changes create defunct protein, in heterozygous mutations the wild-type $TP53$ in the other chromosome can salvage the normal function. In DNA contact mutation, the protein appears to be normal, but cannot exert its normal effect on DNA. This can lead to down-regulation of normal p53 in the cell (Weisz, Oren, & Rotter, 2007). The mutations in residues 175, 245, 248 and 273 are DNA-binding domain mutations. In mouse models mutation at residues 172 and 270 increase carcinogenetic potential. This carcinogenic effect has been seen even in those mice with functioning p53 remaining. An increase in carcinogenic potential was observed in comparison with those mice with null p53. The above-mentioned residues are synonymous to human residues 175 and 273 (Lang et al., 2004; Olive et al., 2004). The amino acid substitution in mutated protein also seems to play a role in carcinogenic potential. At residue 273, different amino acid substitutions confer different responses, as $TP53$ mutants R273H and R273C enhance cancer cell malignancy, but amino acid substitution R273G does not (J. Li et al., 2014). As p53 has many roles in normal biology, p53 mutants have many different outcomes in relation to those roles. Some mutations can exert negative regulation of downstream targets and other mutations can activate pathways which promote cell survival and thus progression of cancer.

### 2.6.4 Lymphomas and haematological malignancies

Cancers of haematopoietic origin are least affected by p53 mutation. In all haematopoietic neoplasms 10 to 20% have a p53 mutation. Although these mutations are rare events in haematopoietic malignancies, there seems to be a connection between worse survival and p53 mutation (Peller & Rotter, 2003; Preudhomme & Fenaux, 1997). In solid cancers p53 mutation is often a driver of tumorigenesis, but in haematological malignancies several other factors are involved, e.g. Bcl-2.
In HL, p53 point mutation frequency in Reed–Stenberg cells is very low, i.e. under 10% (Elenitoba-Johnson, Medeiros, Khorsand, & King, 1996), but higher frequencies have been observed (Kupper et al., 2001). In FL, mutation frequency is also low, but in some studies some larger mutation numbers have been observed. O’Shea et al. analysed point mutations in 185 patients with de novo FL and only 12 heterozygous point mutations were discovered; thus mutation frequency was 6% (O’Shea et al., 2008). In chronic lymphocytic leukaemia deletion of 17p is a known risk factor of poor prognosis and p53 mutation seems to enhance it. In a study by Dicker et al. mutation frequency was 13.5% (26 patients) in patient material of 193 cases. Mutation was associated with deletion of 17p and predicted a short median time to treatment (Dicker et al., 2009). This result was confirmed in later studies with larger cohorts of patients, but single mutation did not predict survival as in the study by Dicker et al. (Jeromin et al., 2014; Stengel et al., 2017). In DLBCL the frequency of TP53 mutation is about 20%. TP53 mutations, especially in LSH and L3 motifs have been associated with poor prognosis (Xu-Monette et al., 2012; Young et al., 2008). Other haematological malignancies have also been studied, but only on a small scale, so conclusions cannot be drawn (Cheung, Horsman, & Gascoyne, 2009).

2.7 Transcription regulators

MYC is an important regulator of transcription and normal function is integral to normal cells. If constitutionally activated it causes over-expression of many proteins that regulate survival and cell growth. It is most known for translocation and constitutive activation in BL (Meyer & Penn, 2008). Bcl-2 and Bcl-6 are both important in normal lymphocyte development, but both are most known for activation in lymphomas.

2.7.1 MYC

MYC is transcriptional regulator of 10–15% of all genes. It regulates histone acetyltransferases, which, through modification of chromatin folding, affect the transcription of genes (Cotterman et al., 2008; Frank et al., 2003; McMahon, Wood, & Cole, 2000a). MYC resides in chromosome 8.

MYC was originally discovered over 35 years ago (Neel, Hayward, Robinson, Fang, & Astrin, 1981). Its function was a conundrum, as no mutation in the gene was discovered, but instead over-expression of normal protein had an oncogenic
effect. The first clue came after insertion of a viral promoter region adjacent to the MYC coding region. The promoter region caused constitutive activation of MYC (Steffen, 1984). Chromosomal translocations were discovered earlier in neoplasms and were connected to oncogenesis, but the reason remained unclear until MYC was mapped to chromosome 8. This discovery led to understanding the significance of translocation in BL. In BL, translocation of an immunoglobulin promoter region adjacent to a MYC coding region causes increased MYC activation, similarly to the viral promoter (Taub et al., 1982). In a mouse model MYC coupled to an immunoglobulin enhancer caused tumour formation originating from both mature and immature B-cells (Adams et al., 1985). In addition to these two mechanisms, amplification of the MYC coding region is a known activation pathway (Alitalo, Schwab, Lin, Varmus, & Bishop, 1983; Dalla-Favera, Wong-Staal, & Gallo, 1982). This amplification occurs normally only during development, but in neoplasms it can be present and is often associated with poor prognosis, e.g. in neuroblastoma (Brodeur, Seeger, Schwab, Varmus, & Bishop, 1984; Tsuchida et al., 1996), breast cancer (Gogas et al., 2016) and ovarian neoplasms (Tafe et al., 2016). In some neoplasms amplification does not affect prognosis, e.g. in DLBCL (Landsburg et al., 2016). Translocation is common in haematopoietic neoplasms and amplification is more often present in solid tumours.

MYC functions both as a transcriptional repressor and activator through binding to DNA, and histone modification. Binding to DNA is possibly through a helix-loop-helix structure in MYC and a heterodimer with MYC partner protein MAX (Blackwood & Eisenman, 1991). MYC regulates chromatin structure through several different proteins, e.g. transactivation/transformation-associated protein (McMahon, Van Buskirk, Dugan, Copeland, & Cole, 1998; McMahon, Wood, & Cole, 2000b) and INI1 (Cheng et al., 1999). MYC in the light of current knowledge binds to 10–15% of all promotor regions in the genome and regulation of MYC is complex (Meyer & Penn, 2008). MYC is the downstream activator of many different activation pathways including PI3K and many pathways are activated through MYC (Hartl, 2016).

MYC-targeting drugs have been thought to be difficult or even impossible to create as a result of a lack of small-molecule interactions and enzymatic function. Antibody-based drugs are also non-functional due to the nuclear localization of MYC (Posternak & Cole, 2016). However, MYC–MAX interaction is an integral part of MYC function and it is possible to interfere with this interaction with direct small-molecule inhibitors. In in vitro experiments KJ-Pyr-9 seems
promising as it reduces MYC-driven growth in MYC-amplified cancer cells (Hart et al., 2014; Raffeiner et al., 2014). Indirect inhibition of cyclin-dependent kinase 7 seems to block MYC-associated growth and kills MYC-driven tumours, e.g. triple-negative breast cancer (Y. Wang et al., 2015) and small-cell lung cancer (Christensen et al., 2014). In a lymphoma cell line dependent on MYC function, disruption of MYC function by eukaryotic initiation factor 4F complex assembly was lethal in premalignant B-cells (C. J. Lin et al., 2012). Much development and further study into intricate molecular mechanisms is required to develop a functioning compound.

2.7.2 Bcl-2

Bcl-2 is a regulator of apoptosis. It is highly conserved among species, as human Bcl-2 in the nematode Caenorhabditis elegans reduced apoptosis (Vaux, Weissman, & Kim, 1992). This conservation underlines its significance. It is also an integral part of B-cell development. Though transient activation it protects early B-cells from apoptosis. Bcl-2 functions by inhibition of pro-apoptotic molecules BAX and BAK. These molecules cause mitochondrial outer-membrane permeability, which leads to release of cytochrome c into the cytosol, which in turn leads to activation of caspase 9 and the caspase cascade through apoptotic peptidase activating factor 1. The caspase cascade causes apoptosis (Delbridge, Grabow, Strasser, & Vaux, 2016; D. R. Green & Kroemer, 2004).

The first hints of the action of Bcl-2 were discovered in non-Burkitt’s lymphoma, as translocation of chromosome 14 was associated with a poorly differentiated lymphoma labelled FL in modern pathology (Fukuhara & Rowley, 1978). Later the gene was cloned and identified in chromosome 18. As Bcl-2 was closely associated with FL it was suggested that it was an oncogene, but studies into the function of Bcl-2 quickly dismissed this idea. In a milestone study by Vaux et al., Bcl-2 was discovered to potentiate the oncogenic effect of c-myc in mice. In the same study Bcl-2 alone did not affect the growth factor needs in an interleukin 3 (IL-3)-dependent lymphoid and myeloid cell line, i.e. it did not cause proliferation as oncogenes do, but promoted survival in the absence of IL-3 (Vaux, Cory, & Adams, 1988). In a later study with transgenic mice, pro-survival effects of Bcl-2 were reaffirmed, as Bcl-2 translocation caused increased B-cell survival and lymphoproliferation in the follicles of germinal centres (McDonnell et al., 1989). In a murine model with combined translocation of Bcl-2 and MYC, mice developed novel lymphomas and plasmacytomas that were early in B cell
development. As these malignancies were early in development, it showed the striking oncogenic potential of combined insults (Strasser et al., 1990). As studies increased it became apparent that Bcl-2 protected cells from a wide array of cytotoxic stimuli, e.g. anti-cancer drugs (McDonnell et al., 1989; Sentman, Shutter, Hockenbery, Kanagawa, & Korsmeyer, 1991), but it does not protect cells from all apoptotic signalling, e.g. T-cell-mediated (Vaux, Aguila, & Weissman, 1992) and FAS-mediated (Strasser, Harris, Huang, Krammer, & Cory, 1995). Mice with homozygous knockout of Bcl-2 could complete embryonic development, but were growth-retarded and had postnatal mortality. Lymphocyte differentiation was normal, but due to increased apoptosis the thymus and spleen were atrophic. The kidneys were polycystic and the mice had early renal failure (Kamada et al., 1995; Veis, Sorenson, Shutter, & Korsmeyer, 1993).

Several cancers show disturbed mechanisms of Bcl-2 function, most notably FL, with constitutive activation due to t(14;18) translocation. Approximately 20–30% of cases of DLBCL have t(14;18) translocation (Weiss, Warnke, Sklar, & Cleary, 1987), and chromosomal amplifications of Bcl-2 have also been detected (Monni et al., 1997). In CLL microRNA levels of miR-15a and miR-16-1 are down-regulated through deletions or other means. Expression of Bcl-2 has been shown to be inversely correlated with these microRNAs and therefore it is believed that they inhibit Bcl-2 (Cimmino et al., 2005). Due to the aforementioned, several drugs against Bcl-2 have been suggested (Konopleva et al., 2008; Lei et al., 2006; Lessene, Czabotar, & Colman, 2008; Reed et al., 1990), the most promising of these being venetoclax, a BH3 mimetic that inhibits Bcl-2. Venetoclax shows great promise in the treatment of CLL, with high tolerability and high response rates even among patients with relapsed and high-risk disease (Roberts et al., 2016; Stilgenbauer et al., 2016). Studies regarding other lymphoid malignancies are still unfinished. However, cell experiments suggest that synergistic blocking of MYC and Bcl-2 might be an effective treatment strategy in double- or triple-hit lymphomas (Cinar et al., 2015).

2.7.3 Bcl-6

Bcl-6 is an antiapoptotic transcription factor. It was first discovered in DLBCL through analysis of the frequently translocated chromosomal region 3q27 (Baron et al., 1993; Ye et al., 1993). It is most commonly detected in DLBCL (approximately 40% of cases) and is present in 5–10% of cases of FL (Basso & Dalla-Favera, 2012; Lo Coco et al., 1994).
The normal function of Bcl-6 is to regulate the development of B-cells and it is integral in naïve B-cell development and formation of GCs. Naïve B-cells which have a functioning B cell receptor (BCR) are selected to undergo somatic hypermutation and class switch recombination of immunoglobulins. This process is vital to improve the affinity of the antigen and diversity in immunoglobulins, but as the process requires DNA breaks the cells must be made to tolerate DNA damage or risk apoptosis. In this, Bcl-6 comes into play as it increases tolerance to DNA damage, thus allowing the aforementioned processes to occur. As these DNA breaks might create oncogenic changes, the regulation of Bcl-6 is tightly controlled (Basso & Dalla-Favera, 2012). If sufficient DNA damage occurs, Bcl-6 is inactivated by phosphorylation. Phosphorylation and further degradation prevents potentially oncogenic mutations (Phan, Saito, Kitagawa, Means, & Dalla-Favera, 2007). In later stages of B-cell development, when B-cells are introduced to their specific antigen, inactivation of Bcl-6 occurs though phosphorylation by mitogen-activated protein kinase (MAPK) (H. Niu, Ye, & Dalla-Favera, 1998). Bcl-6 functions as a transcriptional repressor of several pathways, e.g. NFkB and MAPK pathways (H. Niu et al., 1998; Saito et al., 2007). One of the most important functions of Bcl-6 is to suppress p53 expression. This suppression occurs by direct binding into the TP53 promoter region (Phan & Dalla-Favera, 2004).

As the major role of Bcl-6 is to facilitate the development of B-cells, problems occur when the supposed transient activation turns into constant activation though mutation or translocation. Although the exact mechanisms behind Bcl-6-related lymphomagenesis are still uncovered it is theorized that Bcl-6 translocation grants a survival advantage. Due to the survival advantage the accumulation of additional mutations is possible and therefore further development into lymphoma is possible. This process has been shown in a mouse model. Mice that harboured constitutive activation of Bcl-6 developed lymphoma with features typical of human DLBCL (Cattoretti et al., 2005).

In DLBCL heterogeneity exists in Bcl-6 aberrations. Bcl-6 mutations are more common in GCB DLBCL (70% vs. 44%) and Bcl-6 translocation in ABC (24% vs. 10%). Patients with higher Bcl-6 mRNA or protein levels fare better than those without (Iqbal et al., 2007). Overall, the role of Bcl-6 in development of DLBCL is still uncovered, but evidence that links it to lymphomagenesis has been presented. Currently there are no drugs to affect Bcl-6, but some potential therapeutic synergism with Bcl-2 drugs has been shown (Dupont et al., 2016).
3 Aims of the present study

DLBCL and FL are both neoplasms that have a reasonably good survival. However, there are patients with aggressive disease that succumb to the illness. Currently there are only a few biological markers that can be used to discover the patients that would require more aggressive treatment in first-line therapy. In solid neoplasms connections between survival and oxidative stress have been established, but oxidative stress enzymes are largely unstudied in lymphomas. Only a few studies have been presented of oxidative stress and DLBCL in the pre-rituximab era.

TP53 mutations are among the most common mutations in aggressive neoplasms and a few studies have shown that there is connection between survival and TP53 status in DLBCL. MYC, Bcl-2 and Bcl-6 translocations are often discovered in very aggressive lymphomas. Currently there are limited studies concerning these factors.

The specific aims of the studies were:

1. To evaluate the effect of redox-state-regulating enzymes and oxidative stress markers in patients with DLBCL treated with R+CHOP-like.
2. To evaluate the effect of redox-state-regulating enzymes and oxidative stress markers in patients with FL.
3. To evaluate the effect of TP53 mutations and MYC, Bcl-2 and Bcl-6 translocation in patients with DLBCL treated with R+CHOP-like.
4 Materials and methods

4.1 Patient population and methods (I–III)

The patient population was collected and detailed patient information collected from the University Hospital of Oulu for Articles I–III, the University Hospital of Kuopio for Articles I and III, the University Hospital of Helsinki for Article II and Jyväskylä Hospital for Article III. For Article II two fresh and two FL fresh-frozen samples were obtained from patients, with written consent. The fresh tissue samples were used for immunoelectron microscopy. The overall patient population in Article I was 106 patients with histologically confirmed DLBCL. In Article II the patient population was 79 untreated patients with histologically confirmed FL. The patient population of Article III was 155 patients with histologically confirmed untreated DLBCL. Table 7 summarizes the methods used in Articles I–III. Patient characteristics are summarized in Table 8.

Table 7. Methods

<table>
<thead>
<tr>
<th>Methods</th>
<th>Article I</th>
<th>Article II</th>
<th>Article III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunoelectron microscopy</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>FISH</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Mutation analysis</td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Statistical analysis</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

4.1.1 Details of collected information (I–III)

Patient information was collected in 1998–2013. Initial diagnosis was carried out by histopathological immunostaining of samples taken from lymph nodes or extralymphatic organs of tumour sites. This analysis was done by clinical pathologists and confirmed later by an experienced haematopathologist (Jan Böhm (III), Marja-Liisa Karjalainen-Lindsberg (II) and Kirsi-Maria Haapasaari (I-III)) in each of the hospitals. Before treatment, physical examination, blood work, patient history, examination of tissue samples, bone marrow biopsy and aspiration sample and imaging by computer tomography, full stomach ultrasonography and/or thoracic X-ray were performed. The following information was collected: date of diagnosis, date of birth, age at diagnosis, gender, grade of the disease in FL, stage of the disease (Ann Arbor), B symptoms,
IPI score and variables, FLIPI score and variables if FL, treatment protocol, last follow-up date, date of relapse or progression, date of death and primary treatment.

Table 8. Patient characteristics

<table>
<thead>
<tr>
<th>Article Parameters</th>
<th>I (n/%)</th>
<th>II (n/%)</th>
<th>III (n/%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnosis</td>
<td>108 / 100</td>
<td>0</td>
<td>155 / 100</td>
</tr>
<tr>
<td>FL</td>
<td>0</td>
<td>76 / 100</td>
<td>0</td>
</tr>
<tr>
<td>Histological grade</td>
<td>1</td>
<td>30 / 40</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>34 / 45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3A</td>
<td>12 / 16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GC</td>
<td>36 / 33</td>
<td>56 / 36</td>
<td></td>
</tr>
<tr>
<td>Median age (yr)</td>
<td>65</td>
<td>57</td>
<td>64</td>
</tr>
<tr>
<td>Gender</td>
<td>Female</td>
<td>51 / 47</td>
<td>37 / 49</td>
</tr>
<tr>
<td>B-symptoms</td>
<td>39 / 36</td>
<td>17 / 22</td>
<td>62 / 40</td>
</tr>
<tr>
<td>Stage</td>
<td>III–IV</td>
<td>57 / 53</td>
<td>53 / 70</td>
</tr>
<tr>
<td>IPI</td>
<td>0–1</td>
<td>34 / 32</td>
<td>40 / 53</td>
</tr>
<tr>
<td>2–3</td>
<td>46 / 43</td>
<td>33 / 44</td>
<td>75 / 48</td>
</tr>
<tr>
<td>4–5</td>
<td>22 / 20</td>
<td>3 / 4</td>
<td>18 / 11</td>
</tr>
<tr>
<td>FLIPI</td>
<td>0–1</td>
<td>26 / 34</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>21 / 28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3–5</td>
<td>29 / 38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rituximab</td>
<td>Yes, 1st line</td>
<td>108 / 100</td>
<td>66 / 90</td>
</tr>
<tr>
<td></td>
<td>Yes, 2nd line or later</td>
<td>0</td>
<td>5 / 7</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>0</td>
<td>3 / 3</td>
</tr>
<tr>
<td>Radiotherapy</td>
<td>Yes</td>
<td>58 / 54</td>
<td>8 / 11</td>
</tr>
<tr>
<td>Median follow-up (mo.)</td>
<td>35</td>
<td>91</td>
<td>47</td>
</tr>
<tr>
<td>Relapse</td>
<td>No</td>
<td>89 / 82</td>
<td>38 / 50</td>
</tr>
<tr>
<td></td>
<td>Yes, confirmed</td>
<td>19 / 18</td>
<td>38 / 51</td>
</tr>
<tr>
<td>Mortality</td>
<td>Death from lymphoma</td>
<td>12 / 11</td>
<td>8 / 11</td>
</tr>
<tr>
<td></td>
<td>Death from other causes</td>
<td>6 / 6</td>
<td>6 / 8</td>
</tr>
<tr>
<td>Transformation</td>
<td>0</td>
<td>6 / 8</td>
<td>0</td>
</tr>
</tbody>
</table>
Primary treatment for all DLBCL patients was CHOP-like treatment with rituximab, *i.e.* CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone), CHOEP (CHOP + etoposide) and CEOP (cyclophosphamide, epirubicin, vincristine and prednisone). Treatment for FL was also CHOP-like regimens. Treatments were administered in 21-day cycles. Involved-field radiotherapy was used for low-stage FL as primary treatment and also if DLBCL masses were considered as bulky disease (up to 40 Gy; 2 Gy per fraction and 5 fractions per week). CNS prophylaxis was given to DLBCL patients with IPI more than 3 and primary involvement of testis, breast or base of skull and paraspinal tumour location.

4.2 Immunohistochemistry (I–III)

Primary antibodies are summarized in Table 9. Immunohistochemical samples from patients were obtained from diagnostic biopsies, which were acquired before treatment. These samples were first fixed in formalin and then embedded in paraffin. Paraffin blocks were cut into 3-μm sections and then placed on SuperFrostPlus glass slides (Menzel-Gläser, Braunschweig, Germany). The slides were incubated at +37 °C for 4 hours (Studies I and II) and then deparaffinized using a clearing agent (Histo-Clear; National Diagnostics, Atlanta, GA, USA). Next, the slides were rehydrated in a descending ethanol series. Antigens were retrieved using a microwave oven with the help of sodium citrate buffer, pH 6. Using a 3% H₂O₂ solution, endogenous peroxide activity was blocked. The primary antibodies were used and staining methods performed according to the manufacturers’ instructions. These are presented in Table 7. When using the Vectastain Elite ABC kit, slides were washed with phosphate-buffered saline (PBS) and with other kits, with Tris-buffered saline (TBS) at different stages of staining. At the end of staining, the slides were counterstained with haematoxylin, dehydrated and mounted with Histomount (National Diagnostics, New Jersey, USA). Previously known positive control samples were included in the series. Negative controls were treated with TBS or PBS instead of primary antibody.

Staining were evaluated in a multiheaded microscope by Pekka Peroja (I-III), Kirsi-Maria Haapasaari (I-III) and Laura Lilja (I). Immunohistochemical staining was evaluated by counting the positive and negative lymphoma cells. Percentages were evaluated with 10% margins and <5% was considered to be negative. Intensity was evaluated in each case separately with the aid of negative and
strong-positive controls. Each sample was given an intensity score: 0 = negative, 1 = weak, 2 = intermediate, and 3 = strong.

In Study II, a combination variable from Prx intensities was created by adding all Prx (I–VI) intensities in a single case. The Prx combination variable ranged from six to a maximum of 18, with the use of 11 as an optimal cut-off point. The cut-off point was established manually, using death from lymphoma as an optimal marker. The cases were classed as high Prx or low Prx.

Table 9. Immunohistochemical primary antibodies

<table>
<thead>
<tr>
<th>Study</th>
<th>Molecule</th>
<th>Product ID</th>
<th>Dilution</th>
<th>Source of antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>8-OHdG</td>
<td>MOG020P</td>
<td>1.50</td>
<td>JaICA, Nikken SEIL Co., Ltd,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Fukuroi, Shizuoka, Japan</td>
</tr>
<tr>
<td>I</td>
<td>GCL</td>
<td>sc-22755</td>
<td>1.100</td>
<td>Santa Cruz Biotechnology, Inc.,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Santa Cruz, CA, USA</td>
</tr>
<tr>
<td>I</td>
<td>MnSOD</td>
<td>S5069</td>
<td>1:1000</td>
<td>Sigma-Aldrich, Inc., St. Louis, MO,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>USA</td>
</tr>
<tr>
<td>I-II</td>
<td>Nitrotyrosine</td>
<td>06-284</td>
<td>1:2000</td>
<td>Upstate (Millipore), New York, NY,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>USA</td>
</tr>
<tr>
<td>I-II</td>
<td>Trx</td>
<td>705</td>
<td>1:1000</td>
<td>American Diagnostica, Inc.,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Stamford, CT, USA</td>
</tr>
<tr>
<td>I-II</td>
<td>Prx I</td>
<td>LF-MA0031</td>
<td>1:100</td>
<td>AbFrontier, Seoul, South Korea</td>
</tr>
<tr>
<td>I-II</td>
<td>Prx II</td>
<td>LF-MA0144</td>
<td>1:8000</td>
<td>AbFrontier, Seoul, South Korea</td>
</tr>
<tr>
<td>I-II</td>
<td>Prx III</td>
<td>LF-MA0043</td>
<td>1:2500</td>
<td>AbFrontier, Seoul, South Korea</td>
</tr>
<tr>
<td>I-II</td>
<td>Prx IV</td>
<td>LF-MA0006</td>
<td>1:2000</td>
<td>AbFrontier, Seoul, South Korea</td>
</tr>
<tr>
<td>I-II</td>
<td>Prx V</td>
<td>LF-MA0002</td>
<td>1:500</td>
<td>AbFrontier, Seoul, South Korea</td>
</tr>
<tr>
<td>I-II</td>
<td>Prx VI</td>
<td>LF-MA0013</td>
<td>1:1000</td>
<td>AbFrontier, Seoul, South Korea</td>
</tr>
<tr>
<td>III</td>
<td>MYC</td>
<td>ab32072</td>
<td>1:100</td>
<td>Epitomics, Burlingame, CA, USA;</td>
</tr>
<tr>
<td>III</td>
<td>Bcl-2</td>
<td>M0887</td>
<td>1:100</td>
<td>Dako, Glostrup, Denmark</td>
</tr>
<tr>
<td>III</td>
<td>Bcl-6</td>
<td>IS625</td>
<td>RTU</td>
<td>Dako, Glostrup, Denmark</td>
</tr>
</tbody>
</table>

4.3 Immunoelectron microscopy (II)

The same antibodies were used as in Article II (Immunohistochemical methods). The four samples were fixed in 0.1 M phosphate buffer with 4% paraformaldehyde. The process continued with immersion in 2.3 M sucrose in PBS. After pre-handling the samples were frozen in liquid nitrogen and cut with a Leica EM UC7 cryoultramicrotome (Leica Microsystems, Vienna, Austria). Next, grids were incubated in 2% gelatine in PBS for 20 min and after that in 0.1% glycine-PBS for 10 min; next was blocking-agent incubation with 1% BSA.
(bovine serum albumin) in PBS for 5 min. PBS with 1% BSA was also later used in washing of samples, and dilutions. Selected areas were exposed to primary antibody for 45 min. The process was continued by incubation with protein A-gold (10 nm) for 30 min (Slot JW & Geuze HJ, 1985). Negative controls were acquired by way of the same procedure, but the primary antibody was replaced with PBS. After that, the samples were stained with neutral uranyl acetate (UA). Next, the samples were embedded in 2% methyl cellulose containing 0.4% UA. The samples were examined and analysed by using a Tecnai Spirit transmission electron microscope (FEI, Eindhoven, the Netherlands). A Quemesa CCD camera was used for image capture (Olympus Soft Imaging Solutions GMBH, Munster, Germany).

4.4 Fluorescence in situ hybridisation (III)

Fluorescence in situ hybridization (FISH) analyses were performed in Study III, using previously constructed tissue microarray samples. The following FISH probes were used according to the manufacturers’ recommendations: BCL2 FISH DNA Probe, Split Signal, Code Y5407, Dako, Denmark; BCL6 Breakapart probe, LPH 035, Cytocell, United Kingdom; MYC FISH DNA Probe, Split Signal, Code Y5410, Dako, Denmark. 100 nuclei were assessed in a high-powered field (×60) for each probe set, whenever possible. Only non-overlapping nuclei were analysed and only those nuclei with sufficiently bright signals were accepted.

4.5 Microdissection (III)

Paraffin-embedded tissue sections were used to extract DNA. The paraffin blocks were cut into 10-µm thick slides. The slides were mounted on polyethylene naphthalate (PEN) membrane-coated slides (P.A.L.M. Microlaser Technologies, Germany). The tissue sections were analysed by experienced haematopathologists (Jan Bohm and Kirsi-Maria Haapasaari) and tumour areas were marked. These marked areas were dissected into smaller pieces using the P.A.L.M. Robot-microlaser system (P.A.L.M. Microlaser Technologies). Samples for DNA isolation were obtained with the assistance of pressure catapulting according to the manufacturer’s instructions.
4.6 TP53 mutation analysis (III)

Tumour-derived DNA was directly sequenced (ABI3130 Genetic Analyzer, Applied Biosystems) for TP53 exons 5, 6, 7 and 8, and two sets of primers were used for exons 5 and 8 to reduce the PCR product sizes (<200 bp). Sequence information was obtained from the NCBI public database (NG_017013.2, NM_000546.5). The primer pairs used are shown in Table 8. PCR amplification was performed with AmpliTaq-Gold® (Applied Biosystems) and in BigDye terminator v.1.1 cycle sequencing reactions (Applied Biosystems). The sequencing reactions were performed in forward and reverse directions. Unclear or otherwise unsuccessful samples were re-sequenced. Purification of PCR products was performed by way of ExoSAP-IT® (Affymetrix) or ExoStar™ (Illustra) one-step cleanup reactions with basic ethanol/EDTA precipitation. Sequences were analysed with the assistance of CodonCodeAligner v4.1.1 (CodoneCode Corporation) and Sequence Scanner v1.0 (Applied Biosystems). Mutations were further analysed with the assistance of IARC database version R18, April 2018 (Bouaoun et al., 2016).

Table 10. PCR primers for TP53 exons 5, 6, 7 and 8 (III)

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5'-3'</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex5A forward</td>
<td>CCTGACTTTCAACTCTGTCTC</td>
<td>158 bp</td>
</tr>
<tr>
<td>Ex5A reverse</td>
<td>ACTGCTTGTAGATGGCCATG</td>
<td></td>
</tr>
<tr>
<td>Ex5B forward</td>
<td>ACTGCTTGTAGATGGCCATG</td>
<td>182 bp</td>
</tr>
<tr>
<td>Ex5B reverse</td>
<td>CTGGGGACCTGGGCAAC</td>
<td></td>
</tr>
<tr>
<td>Ex6 forward</td>
<td>GCCCTCTGATTCCTCACTGAT</td>
<td>181 bp</td>
</tr>
<tr>
<td>Ex6 reverse</td>
<td>TTAACCCCTCCTCCAGAGA</td>
<td></td>
</tr>
<tr>
<td>Ex7 forward</td>
<td>AGGCCACCTGGCCATCTT</td>
<td>177 bp</td>
</tr>
<tr>
<td>Ex7 reverse</td>
<td>TGTGCAGGGTGGCAAGTGGC</td>
<td></td>
</tr>
<tr>
<td>Ex8A forward</td>
<td>CCTTA CTGCCTCTTGCTTCTC</td>
<td>130 bp</td>
</tr>
<tr>
<td>Ex8A reverse</td>
<td>CTTGCGGAGATTCTCTCCTC</td>
<td></td>
</tr>
<tr>
<td>Ex8B forward</td>
<td>TTGTGCCCTGTCCCTGGAGAG</td>
<td>127 bp</td>
</tr>
<tr>
<td>Ex8B reverse</td>
<td>CTTCCACCGCTTCTTGTCCT</td>
<td></td>
</tr>
</tbody>
</table>

4.7 Statistical analysis (I–III)

Pearson’s 2-sided chi-square test was used to test for associations between studied proteins and clinical factors. To analyse survival rates Kaplan–Meier analyses were used and to determine the statistical significance the log-rank test was used.
Disease-specific survival (DSS) was defined as time from the date of diagnosis to the date of lymphoma-related death or to the last known follow-up date. Overall survival (OS) was defined as time from the date of diagnosis to death from any cause or last follow-up. Disease-free survival (DFS) was defined as time from the date of diagnosis to the date of relapse or date of death from any cause, whichever occurred first. The patients were censored for analysis at the time of last follow-up. Values of p under 0.05 were considered to be statistically significant. In Study III significant associations with survival observed in univariate analysis were subjected to multivariate analysis with Cox regression using the enter method. Models included IPI divided into three categories per risk (low 0–1, intermediate 2–3 and high risk 4–5), lactate hydrogenase (normal and high), Eastern Cooperative Oncology Group (ECOG) performance status (performance status categories were 0 or 1 and 2, 3 or 4), Ann Arbor stage (Stages I–II and III–IV), age (under 60, and 60 or over), B-symptoms (no and yes), extranodal involvement (no extranodal disease and extranodal involvement), and test variable. The Statistical Package for the Social Sciences, v. 22.0 (IBM SPSS, Chicago, IL, USA) was used perform the analysis.

4.8 Ethical aspects

The principles of declaration of Helsinki were followed throughout the study. The study was approved by the regional Ethics Committee of Northern Ostrobothnia Hospital District (42/2010) and by the National Supervisory Authority for Welfare and Health (Valvira 6622/05.01.00.06/2010).
5 Results

5.1 Oxidative stress staining patterns in DLBCL (I)

In Article I, the redox state was analysed in DLBCL using IHC of GCL, 8-OHdG, MnSOD, Trx and nitrotyrosine. As regards 8-OHdG, nitrotyrosine, Trx and GCL, staining was diffuse and cytoplasmic. The staining pattern of MnSOD was granulated. 8-OHdG and Trx showed diffuse nuclear staining in some malignant cells. There was no detectable nuclear staining for nitrotyrosine, MnSOD or GCL. In some samples staining for 8-OHdG was pronounced at the nuclear membrane. The expression patterns of 8-OHdG and nitrotyrosine were uniform in malignant cells. The staining intensities of Trx, MnSOD and GCL varied in malignant cells in the same sample in a few cases. Staining intensities varied from 0 to 3 and extensities varied from 0 to 100% in 8-OHdG and MnSOD stainings. Nitrotyrosine, Trx and GCL intensities varied from 1 to 3 and extensity was 100% in all cases.

5.2 Oxidative stress staining analysis in DLBCL (I)

Staining patterns in Study I showed only two associations with clinical factors. Extranodal involvement (p = 0.00002) and high IPI scores (p = 0.002) were associated with strong cytoplasmic 8-OHdG staining. The only associations between staining patterns were those of high-intensity cytoplasmic 8-OHdG with high Trx (p = 0.011) and high GCL (p = 0.0003) cytoplasmic intensity.

Staining results were associated with survival. Strong-intensity cytoplasmic Trx staining marked poor PFS (5-year PFS 57.4% versus 85.1%, p = 0.046) and DSS (p = 0.015). Patients with high-intensity cytoplasmic nitrotyrosine staining fared worse than others (5-year PFS 55.7% versus 85.8%, p = 0.006). Those patients with moderate to strong GCL cytoplasmic staining intensity were associated with poor prognosis (5-year PFS 70.8% versus 87.0%, p = 0.049, and 5-year DSS 77.5% versus 94.6%, p=0.062). Staining patterns of MnSOD and 8-OHdG were not associated with survival.

By combining staining patterns and phenotyping, additional groups that had worse survival were discovered. If cytoplasmic Trx or nitrotyrosine immunostaining intensity was strong, patients had worse survival (PFS, p = 0.003 and DSS, p = 0.031) compared with the other patients. Non-GC phenotype
DLBCL with strong cytoplasmic Trx expression was associated with worse survival (5-year PFS 31.6% versus 78.4%, \( p = 0.010 \)). The most important survival plots are presented in Figure 2.

**Fig. 2.** A=Trx cytoplasmic staining divided into negative to intermediate and strong DSS, B=Nitrotyrosine cytoplasmic staining divided into negative to intermediate and strong PFS, C=Strong cytoplasmic staining of nitrotyrosine and/or Trx compared to others
5.3 Oxidative stress staining patterns in FL (II)

In Study II staining patterns varied among the antibodies. Nitrotyrosine, Trx and all Prxs showed cytoplasmic staining. Nuclear staining was observed as regards Trx, Prx I and Prx VI. Prx III staining was more granular than other staining patterns. Trx staining showed three distinct patterns: diffuse, diffuse and spotted, and spotted, the most common being diffuse. Staining of Prxs I and III–VI, and nitrotyrosine in lymphoma cells had 100% extensity and intensity varied between 1 to 3. Negative staining results were observed as regards Prx II and Trx and in these cases extensity varied from 0 to 100% and intensity from 0 to 3.

Immunoelectron labels in Study II showed differences between different Prxs, but no clear differences between FL and hyperplastic lymph node control samples was seen. Prxs I, II, III, V and VI showed labelling in nuclei and cytoplasm. Prx II, Prx III and Prx V showed mitochondrial labelling. Prx I and Prx III labelling had no specific concentrated section in the cells. Prx II was concentrated in the outer membranes of the mitochondria, but not all mitochondria had labels. Prx V mitochondrial staining was concentrated in the cristae. Prx VI labels were concentrated in the cytoplasm close to the plasma membrane and in the nucleus the labelling was concentrated in the vicinity of the nuclear membrane and heterochromatin. Examples of IHC staining are shown on Figure 2. Examples of immunoelectron microscopy is shown on Figure 3.
Fig. 3. Immunohistochemistry staining pattern of FL. A=Trx low intensity, B=prx III strong intensity, C=Prx II strong intensity, D= Prx V low intensity, E=Prx VI low intensity, F=Prx I low intensity
Fig. 4. Immunoelectromicroscope staining patterns FL A=Prx VI staining, B=Prx V staining, C=Prx III staining, D=Prx II staining, E=Prx I staining
5.4 Oxidative stress staining analysis in FL (II)

In Study II staining results showed associations between the peroxiredoxins. Strong Prx III cytoplasmic intensity was associated with high cytoplasmic intensity of Prx I (p = 0.012) and Prx II (p = 0.021). High cytoplasmic intensity of Prx VI was associated with high Prx V cytoplasmic intensity (p = 0.033). High cytoplasmic intensity of nitrotyrosine staining was associated with high staining intensities of several Prxs (Prx I, p = 0.028; Prx II, p = 0.028; Prx III, p = 0.022). Strong cytoplasmic staining of Trx was linked to high-intensity Prx II (p = 0.003) and Prx IV staining (p=0.005). High nitrotyrosine cytoplasmic intensity was associated with high levels of lactate dehydrogenase (p = 0.006). High-level cytoplasmic staining of Prx II and advanced-stage lymphoma (Ann Arbor III–IV) were associated (p = 0.004). No other associations were discovered.

Survival analysis in connection with individual Prxs did not reveal statistically significant prognostic trends, but a combination variable of all Prxs had prognostic power. High total Prx intensity was associated with favourable OS and DSS (5-year OS 100% versus other patients, 83.2%, p = 0.035 and 5-year DSS 100% versus other patients, 83.2%, p = 0.035) (presented in Figure 5), but not with PFS (p = 0.647). Patients with advanced disease (Ann Arbor III–IV) and/or low total Prx intensity had poor outcomes in terms of DSS (5-year DSS 81.5% versus other patients, 100%, p = 0.00051).

Fig. 5. High Prx combination variable versus others DSS
5.5 **TP53 mutations (III)**

Sequencing was successful in 80 samples (total 155). Sequencing was unsuccessful in 75 samples, with 26 samples having unsuccessful sequencing of one exon. In total, 10 different mutations were detected and mutation frequency was 12.5%. Nine of the mutations detected were missense mutations, eight resulted in non-functional p53 and one gave partially functional p53 according to IARC database analysis. One mutation gave a synonymous protein product and therefore the mutation was silent. Three mutations were detected in region DBD (one in L3 and two in LSH); the other seven mutations were in β-sheets. Mutations are presented in Table 11.

Overall, *P53* mutations did not predict survival in our study. Patients with *TP53* mutations had aggressive primary refractory diseases, but no relapses. In more in-depth structural analysis, *TP53* mutations that were localized in regions LSH and L3 predicted survival (3-year DSS 33.3% versus 83.3% in WT *TP53* plus other *TP53* mutations, p = 0.011, and 3-year DFS 33.3% versus 75.8% in WT *TP53* plus other *TP53* mutations, p = 0.027). Multivariate analysis of LSH and L3 mutations were prognostic as regards survival (risk ratio, death to lymphoma 8.779, CI 95% 1.377–55.972, p = 0.022 and risk ratio, disease progression 10.498, CI 95% 1.710–64.449, p = 0.011).

<table>
<thead>
<tr>
<th>Case number</th>
<th>Exon</th>
<th>Mutated protein</th>
<th>Mutation type</th>
<th>Structural Motif</th>
</tr>
</thead>
<tbody>
<tr>
<td>99</td>
<td>5</td>
<td>V157I Val&gt;Ile</td>
<td>missense</td>
<td>β-sheets</td>
</tr>
<tr>
<td>92</td>
<td>5</td>
<td>I162I Ile&gt;Ile</td>
<td>silent</td>
<td>β-sheets</td>
</tr>
<tr>
<td>47</td>
<td>7</td>
<td>Y236C Tyr&gt;Cys</td>
<td>missense</td>
<td>β-sheets</td>
</tr>
<tr>
<td>100</td>
<td>7</td>
<td>C242W Cys&gt;Trp</td>
<td>missense</td>
<td>L3</td>
</tr>
<tr>
<td>86</td>
<td>7</td>
<td>I251L Ile&gt;Leu</td>
<td>missense</td>
<td>β-sheets</td>
</tr>
<tr>
<td>82</td>
<td>7</td>
<td>E268K Glu&gt;Lys</td>
<td>missense</td>
<td>β-sheets</td>
</tr>
<tr>
<td>33</td>
<td>8</td>
<td>G266E Gly&gt;Glu</td>
<td>missense</td>
<td>β-sheets</td>
</tr>
<tr>
<td>105</td>
<td>8</td>
<td>F270S Phe&gt;Ser</td>
<td>missense</td>
<td>β-sheets</td>
</tr>
<tr>
<td>74</td>
<td>8</td>
<td>R273G Arg&gt;Gly</td>
<td>missense</td>
<td>LSH</td>
</tr>
<tr>
<td>40</td>
<td>8</td>
<td>R273H Arg&gt;His</td>
<td>missense</td>
<td>LSH</td>
</tr>
</tbody>
</table>
5.6 Translocation and immunohistochemistry of MYC, Bcl-2 and Bcl-6 (III)

In Study III translocation analysis with FISH was successful in 128 cases (total 155). Translocations of MYC, Bcl-2 and Bcl-6 were detected in 11, 15 and 22 patients respectively. Double-hit translocations were detected in six patients (MYC and Bcl-2, n = 3; MYC and Bcl-6, n = 3). One triple-hit lymphoma was detected. GC phenotype (p = 0.0000002) and age under 60 (p = 0.050) were associated with Bcl-2 translocation. Extranodal disease was associated with DH disease (p = 0.014). Bcl-2 and MYC translocation did not have any associations with clinical data. None of the translocations or DH predicted survival in our data.

In Study III IHC of Bcl-2, Bcl-6 and MYC was evaluated. IHC data and GC and non-GC phenotyping per Hans’ algorithm was available in 141 cases. IHC evaluation of MYC and Bcl-2 was successful in 128 samples and that of Bcl-6 in 129 samples. MYC expression was associated with intermediate IPI (IPI score 1–2, p = 0.007). Bcl-6 expression was associated with GC phenotype (p = 0.011). Neither MYC nor Bcl-6 were associated with survival. Bcl-2 positivity was associated with survival (3-year DFS 58.9% versus 85.9%, p = 0.002, and 3-year DSS 68.0% versus 90.6%, p = 0.002). Double-expressor lymphoma (DEL), i.e. lymphoma with Bcl-2 and MYC positivity, was associated with dismal survival (3-year DFS 57.4% versus 79.1%, p = 0.011, and 3-year DSS 66.5% versus 85.2%, p = 0.038). In patients with non-GC disease DEL had even more prognostic power (3-year DSS 54.5% versus 85.4%, p = 0.013, and 3-year DFS 49.5% versus 76.2%, p = 0.010).

5.7 Concurrent analysis of TP53 mutation, translocation and immunohistochemistry (III)

In Study III concurrent mutation of TP53 and translocation of Bcl-2 was found in two patients. These two patients had aggressive disease with poor response to treatment, mean DSS being three months (p = 0.00000002). TP53 mutations were also present in one MYC-translocated, one Bcl-6-translocated and one DH lymphoma. Expression of MYC in IHC was associated with Bcl-2 translocation (p = 0.002) and MYC translocation (p = 0.030). Bcl-2 IHC expression was associated with Bcl-2 translocation (p = 0.011). Expression of Bcl-6 in IHC was associated with DH lymphoma (p = 0.041) and MYC translocation was associated
with Bcl-6 translocation (p = 0.022). Double-expressor lymphoma was associated with Bcl-2 translocation (p = 0.001).
6 Discussion

6.1 Role of oxidative stress in DLBCL

Oxidative stress has been implicated in the pathogenesis of several different neoplasms, and their treatment responses. Redox-state-regulating enzymes and oxidative stress have been associated in a few earlier studies on DLBCL, with varying outcomes. In our study, high levels of nitrotyrosine, a marker of oxidative stress, GCL, and Trx, a redox-state-regulating enzyme, were associated with poor survival. These findings were among the first as regards evaluation of oxidative stress in DLBCL patients treated with rituximab.

Earlier studies regarding redox-state-regulating enzymes in DLBCL have shown conflicting results. Tome et al. (2005) investigated levels of antioxidant enzymes and redox-state-regulating proteins in patients with DLBCL treated with CHOP-like therapies (Tome et al., 2005). They studied catalase, glutathione peroxidase, MnSOD and Trx. Their results suggested that decreased expression of catalase, glutathione peroxidase and MnSOD and increased expression of Trx were linked to poor prognosis. The authors further created a redox signature score for survival evaluation. Andreadis et al. examined the expression of proteins related to the glutathione family in CHOP-like-treated patients with DLBCL (Andreadis et al., 2007). Their results largely contradicted those of Tome et al., i.e. increased expression of glutathione-family proteins was a marker of poor prognosis.

When comparing our results with those of previous studies, ours were in line with those of Andreadis et al., as in both studies increased expression of redox-state-regulating enzymes marked poor prognosis. In our study Trx had the same association with poor prognosis as reported by Tome et al., although our overall conclusions were different. Treatment protocols were different in both previous studies, so our results are not directly comparable. In a previous study carried out by our group, in which the expression of different oxidative stress markers in several B-cell lymphomas were characterized, high levels of GCL and nitrotyrosine were connected to poor prognosis in cases of DLBCL (RW.ERROR - Unable to find reference:111).

Levels of GSH have been associated with resistance to doxorubicin (Asano, Tsutsuda-Asano, & Fukunaga, 2009) and vincristine (Tsai, Sun, Lu, Cheng, & Chao, 2007). In Article I, increased expression of the rate-limiting enzyme in
production of GSH, GCL, was linked to poor prognosis. Levels of Trx have been connected to increased tolerance to several cytostatic drugs, i.e. cisplatin, mitomycin c, doxorubicin and etoposide (Yokomizo et al., 1995). The drug-resistance association of redox enzymes might explain the poor prognosis of our patients. The drug-resistance association of Trx in DLBCL was studied by Li et al. (C. Li et al., 2012). In their study Trx expression levels were associated with poor survival and they also found that DLBCL cell lines with elevated expression of Trx had more resistance to cytostatic drugs. By using small interfering RNA the protective effect of Trx was inhibited and this inhibition restored sensitivity to doxorubicin.

Molecular signatures have uncovered underlying groups within DLBCL. One of these groups is the oxidative phosphorylation (OxPhos) group (Monti et al., 2005). It is believed that OxPhos DLBCLs harbour genes involved in mitochondrial metabolism and they are insensitive to B-cell receptor-inhibiting stimuli. This OxPhos subtype shows an excess amount of glutathione synthesis and greater mitochondrial energy production. Experimental data shows that interference in glutathione synthesis is selectively toxic to this type (Caro et al., 2012). In Study I, it could be plausible that we identified the OxPhos subtype in IHC analysis of redox enzymes. Combining our results with experimental data, patients with high amounts of redox-state-regulating enzymes could benefit from alternative strategies that interfere with Trx or glutathione systems. If experimental data regarding resistance to B-cell receptor interference in the OxPhos subtype is corroborated in further studies, intervention with ibrutinib and idelalisib would be futile.

The part played by oxidative stress in DLBCL is still in large part a conundrum, but evidence suggests that in some cases oxidative stress-influencing drugs could have added therapeutic potential. Currently the role of oxidative stress in the aetiology of DLBCL is unstudied, but as inflammation and chronic infection has been implicated in DLBCL aetiology, oxidative stress might be a connective factor.

6.2 Redox enzymes and oxidative stress in FL

In Study II our main results showed that total Prx expression had prognostic potential. High combined Prx intensity seemed to be associated with very favourable DSS, but not with PFS. Further, our immunoelectron microscopy study consolidated and confirmed the discovered localization of Prx enzymes in
the cell (Kinnula et al., 2002). Only a very limited amount of studies regarding oxidative stress and FL have been published. The enzyme 8-oxoguanine DNA glycosylase, which repairs oxidative stress damage to DNA was discovered to be lower in FL than in normal germinal centres (Sheehan et al., 2005). Pasanen et al. studied the expression of several redox-state-regulating enzymes in several lymphomas including FL (Pasanen et al., 2012).

High levels of Prx have been associated with aggressive diseases and chemoresistance in solid malignancies (Kalinina et al., 2012; Kubota et al., 2013; Suenaga et al., 2013; Sun et al., 2014). In DLBCL high cytoplasmic Prx VI levels have been associated with poor survival (Kuusisto et al., 2015). However, our study of FL showed contrasting results. The indolent nature of FL might explain the difference. Patients with FL can go into spontaneous remission and they are usually treated only when symptomatic. High levels of redox enzymes could protect FLs from more aggressive mutations and thus keep the disease indolent. Patients with high levels of Prxs showed a similar number of relapses, but fewer disease-associated deaths. This could imply that FL patients might benefit from treatment strategies that reinforce redox-state-regulating enzymes. However, as no confirmatory studies have been published, our finding remains a very interesting discovery. Contradictory to this theory, the transformation rate was not different between the two total Prx expression groups.

6.3 TP53 mutations, MYC, Bcl-2 and Bcl-6 in DLBCL

In Study III, our results showed that TP53 mutations in LSH and L3 regions were associated with dismal survival. Other TP53 mutations and overall mutations in TP53 did not predict survival. Earlier studies regarding TP53 mutations in DLBCL have linked TP53 mutations with poor prognosis (Schiefer et al., 2015; Xu-Monette et al., 2012; Young et al., 2007). Young et al. examined TP53 mutations in a cohort of 477 patients and discovered 102 mutations. In their study TP53 mutations in LSH and L3 regions were associated with poor survival, but patients with mutations at L2 had the same prognosis as those with WT TP53. Overall mutations to the DBD region in TP53 predicted survival. The study was carried out among patients treated with CHOP-like therapies, but without rituximab (Young et al., 2007). Xu-Monette et al. further examined TP53 mutations in a cohort of 506 patients treated with rituximab and CHOP-like therapies. TP53 mutations, but not deletions were associated with worse survival. TP53 mutations in LSH and L3 regions were once
again highlighted as markers of poor prognosis. GC phenotyping did not affect the results (Xu-Monette et al., 2012). Study III results are further strengthened, as both large studies yielded similar results as our smaller one. The reason for poor prognosis of patients with TP53 mutations in LSH and L3 regions is still unknown.

Concurrent analysis of TP53 mutations and translocations of MYC, Bcl-2 and Bcl-6 have been analysed in only one previous study. Schiefer et al. analysed these factors in a cohort of 101 patients. The cases were gathered at a referral centre and 11 patients with primary transformed FL were included. Samples were screened via p53 immunohistochemistry and those samples with over 30% expression were sequenced. A total of 29 different TP53 mutations were found, but deletions were also included as mutations. Patients without any detected alterations had a median OS of 65 months. Patients who were MYC+/Bcl-2+/TP53+ did not achieve median OS during the follow-up period, so survival was very favourable. Patients with combined TP53 mutation and MYC translocation had poor OS of 10 months. Patients with mutated TP53 alone also had short survival (median OS was 12 months). Further mutational analysis was not presented (Schiefer et al., 2015). Our results differ from these, as patients with combined translocation of Bcl-2 and TP53 mutation had poor prognosis with very short OS of three months. Both patients had LSH and L3 mutations. As we discovered only two patients with TP53 mutation with Bcl-2 translocation and our results are in contrast to those of Schiefer et al., further studies should be aimed at discovering the association between translocations and TP53 mutations.

Fiskvik et al. assessed TP53 status by way of immunohistochemistry and combined the data with that from translocation studies. In their study, patients with high-level expression of MYC and Bcl-2 protein, or translocation combined with high-level expression of p53, or deletion in their lymphomas showed poor prognosis (Fiskvik et al., 2015). This immunohistochemical study showed the same trend as in our study, in that combined activation of Bcl-2 and p53 were markers of poor prognosis, as Bcl-2 blocks apoptotic signalling of p53, and mutated p53 often loses the ability to bind to DNA because the mutation is located in the DNA-binding domain. This combined effect could leave lymphoma cells vulnerable to further mutations and decrease apoptotic signalling.

In Study III, DH status did not affect survival (as seen in other studies). This could have been a statistical anomaly due to the small number of translocations detected. Double expressor status and high-level protein expression of Bcl-2 detected by immunohistochemistry was associated with poor prognosis. This
association has been noted in several studies (Abdulla et al., 2016; Horn et al., 2013; Kawamoto et al., 2016; J. Wang, Zhou, Xu, Chen, & Ouyang, 2015). MYC and Bcl-2 analysis in cases of DLBCL are not part of routine pathological analysis. As both of these factors have prognostic potential, as seen in several studies, addition of these (via immunohistochemical tests) might have prognostic impact and in the analysis of new drugs should be considered together with phenotyping. The new revised WHO IV classification requires the use of translocation studies to distinguish double-hit lymphoma from DLBCL.

Overall, the results of Study III confirmed earlier observations regarding the role of TP53 mutations and raised questions as to the association between survival, combined translocations and TP53 mutations. As mutations to TP53 are rare events in DLBCL and not all mutations seem to predict survival, more large studies should be conducted before drawing conclusions. Immunohistochemical analysis of MYC, Bcl-2 and Bcl-6 could be added to the standard protocol in analysis of DLBCL, at least in clinical trials. In the current study translocations and TP53 mutations explained only a small part of relapses and death to lymphoma, with only about 20% of all deaths to lymphoma being associated with these biological changes.

### 6.4 Limitations in material and methods

As all our patient samples were obtained from diagnostic paraffin-embedded tissue specimens and lymphomas often have different cellular populations with varying localisation, our limited sampling could cover only a small part of the total lymphoma mass. This is more of a problem in FL than in DLBCL, because in DLBCL a dominant cell type is most often found in all parts. In FL, dominant cell subgroups can change over time and with disease progression.

Some of the patients were lost to follow-up due to change in residence and for other reasons. Patient material was collected from four different centres and although all had the same standard for patient material collection, a few clinical factors could not be collected in all cases. Althought all patients were treated with R-CHOP-like treatments in articles I and III, there might be small differences in treatment modalities. In article II treatments varied eventhough most were treated with R-CHOP-like chemotherapies.

In Study I, our antibodies were not verified in a larger series and therefore the exact prognostic power is unknown. Our patient material could have been larger and may have included more centres than two. As a method,
immunohistochemistry cannot explain causalities in protein expression between samples. Even though higher intensities might be a result of a higher amount of protein, without confirmatory assays this is not certain. Because other methodologies were not used to verify elevated protein expression, e.g. qPCR, some undefined factor could remain undetected.

In Study II immunohistochemistry was used to assess redox enzymes and therefore the main limitations are as described above. The amount of patient material for electron microscopy was very small – four samples in total, i.e. two FL samples and two controls, and no differences were detected between the controls and lymphoma tissues. If the patient material had been larger, trends could have been observed, but as our results were in line with those of earlier studies, the present results remain confirmatory.

In Study III the choice of paraffin wax-embedded tissue samples as source material largely limited our methods. Paraffin fixation preserves the tissue, but also causes DNA strand breaks. These strand breaks made analysis of mutations very demanding and partly explain our limited achievement. Because of these strand breaks, mechanical analysis of mutation was made impossible. As we selected only four exons of TP53 for analysis, some mutations are left undetected. Microdissection as a method was useful in obtaining material, but as a limitation, the slide was only a small part of the whole sample. Owing to thick slides, it was not possible to distinguish lymphoma tissue with the microdissection system, and instead we had to rely on diagnostic slides to select the sampling places. Because of the small number of detected mutations, analysis of individual mutations remains uncertain.

6.5 Clinical implications

Oxidative stress and the redox state maybe explain part of the drug resistance in cases of DLBCL. If treatments are developed to counteract the redox defences, and if DLBCL cases of OxPhos status are more closely defined, this could change the treatment protocols. If a protective effect of redox-state-regulating enzymes is further confirmed in FL, the life-spans of patients might be improved with the addition of redox enzymes that protect lymphoma cells from further mutations. Although Studies I and II were experimental and no confirmatory studies have been conducted, both these studies present new biological observations regarding oxidative stress in cases of lymphoma. These should be further studied in larger patient populations and cell-culture models. Further studies that study the role of
oxidative stress regulators might yield insight to the role of oxidative stress activation.

*TP53* mutations are prognostically relevant in DLBCL, but cost-effectiveness of their analysis is still far away. If new drugs that target mutated p53, or drugs that increase effectiveness in mutated p53 are discovered, *TP53* mutations could be analysed before treatment. In CLL, chemotherapeutic agents that are effective regardless of p53 state are in clinical use. As the prognostic potentials of MYC, Bcl-2 and Bcl-6 in DLBCL have been presented in several studies, they should be considered in clinical trials as potential factors to be analysed in more detail.
7 Summary

In the present study, the prognostic potentials of different redox enzymes and the oxidative stress marker nitrotyrosine in DLBCL and FL were investigated. In DLBCL the results of the present study also confirmed earlier observations regarding poor prognosis in cases of \textit{TP53} mutations in LSH and L3 and the usefulness of immunohistochemical evaluation of Bcl-2 and MYC. Furthermore, translocation status and \textit{TP53} mutation were evaluated concurrently. In conclusion:

1. Immunohistochemical expression of Trx and nitrotyrosine associate with poor survival in cases of DLBCL.
2. Total Prx expression is associated with good prognosis in patients with FL.
3. \textit{TP53} mutations in regions LSH and L3, Bcl-2 positivity in immunohistochemistry and double-expressor status are associated with adverse outcomes in DLBCL. \textit{TP53} mutations combined with Bcl-2 translocation seems to be associated with poor prognosis.
References


neoplastic progression and treatment. *Biochimica Et Biophysica Acta, 1853*(7), 1658-
1671.

Correia, C., Schneider, P. A., Dai, H., Dogan, A., Maurer, M. J., Church, A. K., . . .
Kaufmann, S. H. (2015b). BCL2 mutations are associated with increased risk of

Cotterman, R., Jin, V. X., Krig, S. R., Lemen, J. M., Wey, A., Farnham, P. J., & Knoepfler,
P. S. (2008). N-myc regulates a widespread euchromatic program in the human
genome partially independent of its role as a classical transcription factor. *Cancer
Research, 68*(23), 9654-9662.

immunohistochemical analysis of antioxidant and glutathione S-transferase enzyme
evels in normal and neoplastic human lung. *Histology and Histopathology, 11*(4),
851-860.

Courtois, S., Verhaegh, G., North, S., Luciani, M. G., Lassus, P., Hibner, U., . . . Hainaut,
P. (2002). DeltaN-p53, a natural isoform of p53 lacking the first transactivation
domain, counteracts growth suppression by wild-type p53. *Oncogene, 21*(44),
6722-6728.

(2013). Poor concordance among nine immunohistochemistry classifiers of cell-of-
origin for diffuse large B-cell lymphoma: Implications for therapeutic strategies.
*Clinical Cancer Research : An Official Journal of the American Association for
Cancer Research, 19*(24), 6686-6695.

MnSOD and GPX-1 and breast cancer risk: A nested case-control study. *BMC Cancer,
6*, 217.

synthase a target for chemoprevention? *Molecular Cancer Therapeutics, 2*(8),
815-823.

(2015). Persistent disparities among patients with T-cell non-hodgkin lymphomas and
B-cell diffuse large cell lymphomas over 40 years: A SEER database review. *Clinical
Lymphoma, Myeloma & Leukemia, 15*(10), 578-85.

targeting of peroxiredoxin 3 as an effective therapeutic approach for malignant

Cunningham, G. M., Roman, M. G., Flores, L. C., Hubbard, G. B., Salmon, A. B., Zhang,
Y., . . . Ikono, Y. (2015). The paradoxical role of thioredoxin on oxidative stress and


Foksiński, M., Kotzbach, R., Szymanski, W., & Oliński, R. (2000). The level of typical biomarker of oxidative stress 8-hydroxy-2'-deoxyguanosine is higher in uterine myomas than in control tissues and correlates with the size of the tumor. *Free Radical Biology and Medicine, 29*(7), 597-601.


105


Lee, H., Mok, K. H., Muhandiram, R., Park, K. -. , Suk, J. -., Kim, D. -., . . . Han, K. -. 
(2000). Local structural elements in the mostly unstructured transcriptional activation 

induces bax/bak-independent activation of apoptosis and cytochrome c release via a 
conformational change in bcl-2. FASEB Journal : Official Publication of the 


331.

(2012). Over-expression of thioredoxin-1 mediates growth, survival, and 
chemoresistance and is a druggable target in diffuse large B-cell lymphoma. 
Oncotarget, 3(3), 314-326.

Downregulation of peroxiredoxin-1 by beta-elemene enhances the radiosensitivity of 
lung adenocarcinoma xenografts. Oncology Reports, 33(3), 1427-1433.

Human Mutation, 35(5), 575-584.

Li, Y., Huang, T. T., Carlson, E. J., Melov, S., Ursell, P. C., Olson, J. L., . . . Epstein, C. J. 
(1995). Dilated cardiomyopathy and neonatal lethality in mutant mice lacking 
manganese superoxide dismutase. Nature Genetics, 11(4), 376-381.

Lin, C. J., Nasr, Z., Premrisriot, P. K., Porco, J. A., Jr, Hippo, Y., Lowe, S. W., & Pelletier, 
J. (2012). Targeting synthetic lethal interactions between myc and the eIF4F complex 

LEP and LEPR polymorphisms in non-hodgkin lymphoma risk: A systematic review 
and pooled analysis. Journal of B.U.ON.: Official Journal of the Balkan Union of 
Oncology, 20(1), 261-268.

(2016). Peroxiredoxin 1 induces inflammatory cytokine response and predicts 
outcome of cardiogenic shock patients necessitating extracorporeal membrane 
oxigenation: An observational cohort study and translational approach. Journal of 
Translational Medicine, 14(1), 114-016-0869-x.

oxidative stress markers in depression. PloS One, 10(10), e0138904.

Lo Coco, F., Ye, B. H., Lista, F., Corradini, P., Offit, K., Knowles, D. M., . . . Dalla-Favera, 
R. (1994). Rearrangements of the BCL6 gene in diffuse large cell non-hodgkin's 


Pryor, W. A. (1988). Why is the hydroxyl radical the only radical that commonly adds to DNA? hypothesis: It has a rare combination of high electrophilicity, high thermochemical reactivity, and a mode of production that can occur near DNA. *Free Radical Biology & Medicine*, **4**(4), 219-223.


Shaver, A. C., Ma, L., Vnenckak-Jones, C., Schwarting, R. S., Fasig, K. J., & Kim, A. S. (2014). Transformation of small B-cell lymphoma into large cell CD30(+), CD4(+), epstein-barr virus-negative lymphoma. *Archives of Pathology & Laboratory Medicine, 138*(8), 1101-1105.


Original Publications


Reprinted according to Biomed Central licence agreement as copyright holder (I). Reprinted with permission from Springer (II).

Original publications are not included in the electronic version of the dissertation.
1442. Sundquist, Elias (2018) The role of tumor microenvironment on oral tongue cancer invasion and prognosis
1449. Kajula, Outi (2018) Periytyvän riintasyöpäalttiusmutaation (BRCA1/2) kantajamiesten hypoteetettävä perinnöllisyysneuvontamalli
1452. Capra, Janne (2018) Differentiation and malignant transformation of epithelial cells : 3D cell culture models
1453. Panjan, Peter (2018) Innovative microbioreactors and microfluidic integrated biosensors for biopharmaceutical process control
1454. Saarela, Ulla (2018) Novel culture and organoid technologies to study mammalian kidney development
1456. Vuollo, Ville (2018) 3D imaging and nonparametric function estimation methods for analysis of infant cranial shape and detection of twin zygosity

Book orders:
Granum: Virtual book store
http://granum.uta.fi/granum/
Pekka Peroja

OXIDATIVE STRESS IN DIFFUSE LARGE B-CELL LYMPHOMA AND FOLLICULAR LYMPHOMA, AND TP53 MUTATIONS AND TRANSLOCATIONS OF MYC, BCL-2 AND BCL-6 IN DIFFUSE LARGE B-CELL LYMPHOMA